

diSPIM and iSPIM User Manual

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1 Overview

ASI's single plane illumination microscope (SPIM) systems utilize modular mounting and optical components to allow several SPIM variations and operation on various inverted microscope platforms. Common to all systems is an objective mounting arrangement that places two objectives perpendicular to one another. One objective is used to project a light sheet to illuminate the sample and the other objective views emission from the illuminated sample. On dual (diSPIM) systems the two objectives alternately change roles in order to record two perpendicular views of the same object.

The SPIM microscope may be mounted on any number of inverted microscope platforms. Most commonly the SPIM is attached to ASI's modular inverted microscope on a RAMM frame as shown in Figure 1. Adapter brackets are available for several commercial inverted microscopes; the specific microscope mounting bracket will accept the SPIM-MOUNT to which the SPIM microscope assembly attaches.¹ The following microscopes are already supported, and other SPIM brackets can be designed on request:

- Leica DMI-6000
- Nikon TE-300, Ti
- Olympus IX-71/81, IX-73/83
- Zeiss Axio-Observer

The rest of this document will concentrate on the function, alignment and operation of the diSPIM microscope. In a few cases we may explicitly discuss operation of the lower-side microscope focus and camera, which will be different on platforms other than the RAMM frame.

2 SPIM Assembly

The steps in this section are demonstrated in the following YouTube video: <https://www.youtube.com/watch?v=TAgbr6IrTqw>.

2.1 Attach the SPIM assembly to the microscope frame

The SPIM assembly is shipped separately and mostly assembled. The RAMM frame stand or the microscope mount assembly has two vertical arms on the back where 2 dowel pins each (total of 4) connect the microscope frame to the SPIM-MOUNT. Slide the complete SPIM assembly onto the dowel pins and fastened in place with the bolts provided (M8 thread with M6 Allen head). See Figure 2. If you need to set down the SPIM assembly, be careful not to rest it on the piezo objective movers (where the objectives will go) but instead tip it backwards and rest it on the arm and tube lenses to protect the piezos.

Once you have the SPIM assembly attached to the microscope frame, the next step is to add the scanners and cameras to the tube lenses using the C-mount adapters.

2.2 Attach the scanners

Locate the scanner tube lenses, C-mount adapters, and scanners. Attach the scanners to the C-mount adapters as shown in Figure 3. Attach the tube lens assemblies to the lower MIM-CUBE-II's using a 2 mm Allen wrench to secure the three set screws to the adapter ring. Insert the C-mount adapter with scanner attached into the tube lens fitting and secure with the set screws². Be sure the scanner assembly is aligned parallel / perpendicular to the MIM-CUBE-II.

¹Because the brackets utilize the transmitted light pillar mounting connections attaching the SPIM precludes use of the transmitted light optics.

²On some early systems the scanner tube lenses were fixed to the frame, similar to the camera tubes. This is no longer recommended. Some hardware changes may be required to retrofit to the recommended configuration with the scanners fixed to the filter cube; contact ASI for details.

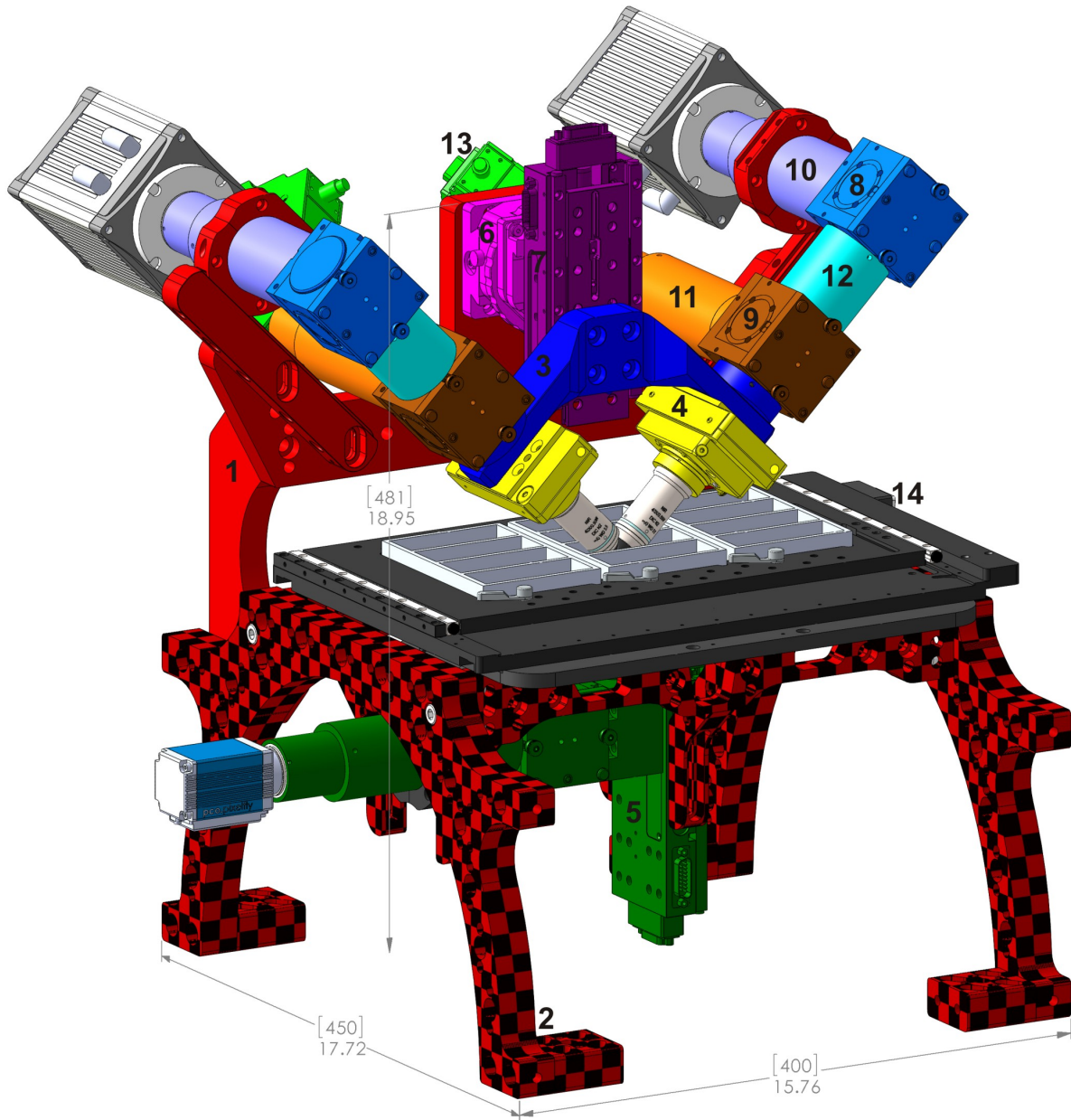


Figure 1: Typical complete diSPIM system on RAMM frame showing major components. 1) SPIM-MOUNT, 2) SPIM-RAMM, 3) SPIM arm mount (RAO-0046), 4) piezo objective mover APZOBJ-200 or similar, 5) MIM inverted microscope with LS-50M stage, 6) CDZ-1000 centering stage,^{††} 7) LS-50M stage, 8) MIM-CUBE-II w/ mirror, 9) MIM-CUBE-II w/ fluorescence filters, 10) C60-TUBE_B imaging tube lens, 11) C60-TUBE_160 scanner tube lens, 12) 50mm extension tube, 13) MM-SCAN_1M light sheet scanner, 14) MS-2500 XY stage. ^{††} On the RAMM frame we prefer to use a solid block for stability instead of the CDZ-1000, and instead co-align the lower objective with the SPIM objectives using the lateral adjusters on the lower objective.

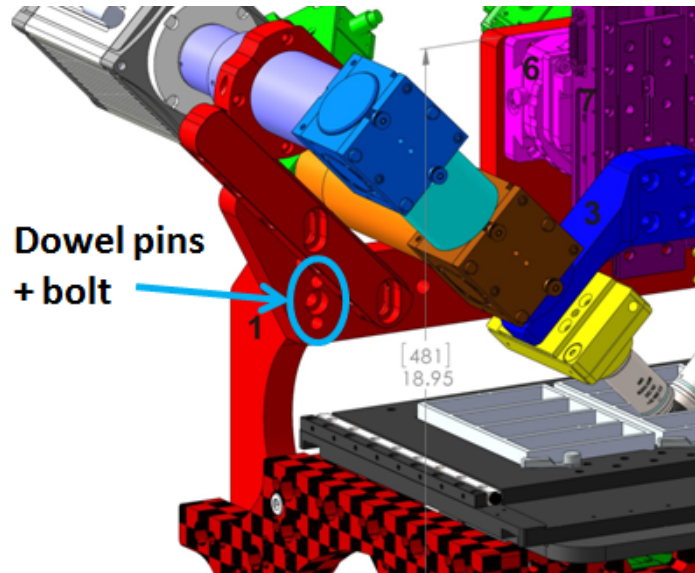


Figure 2: RAMM frame with SPIM assembly, showing location of dowel pins and bolts.

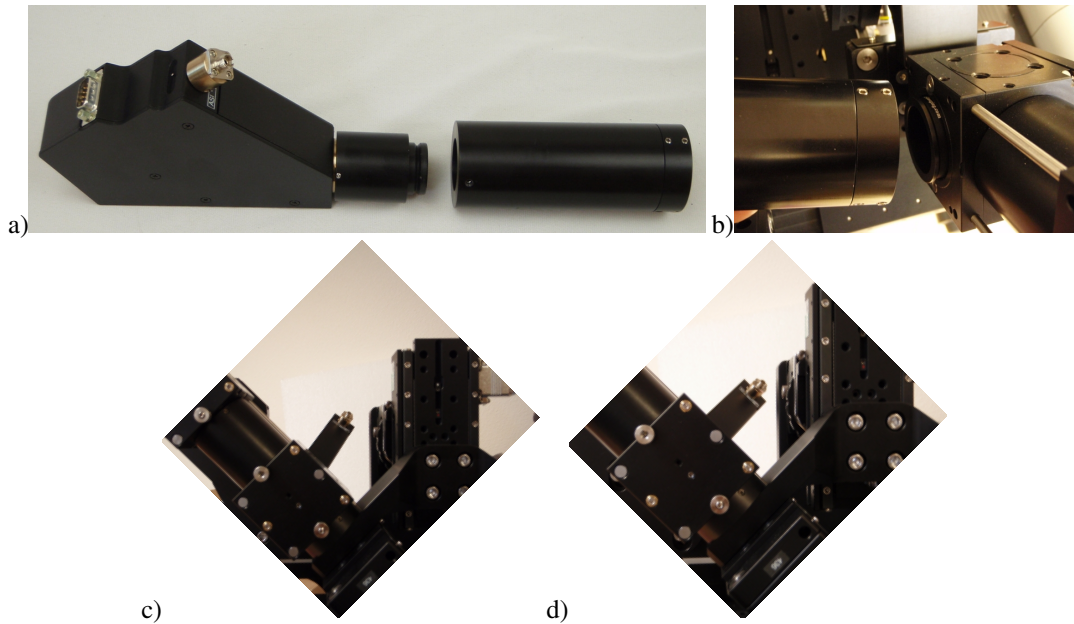


Figure 3: Scanner assembly: a) Scanner attached to C-mount adapter with tube lens assembly ready to be installed. b) Tube lens assembly is attached to lower cubes on the microscope. c) Scanner attached at C-mount with poor alignment; d) better alignment.

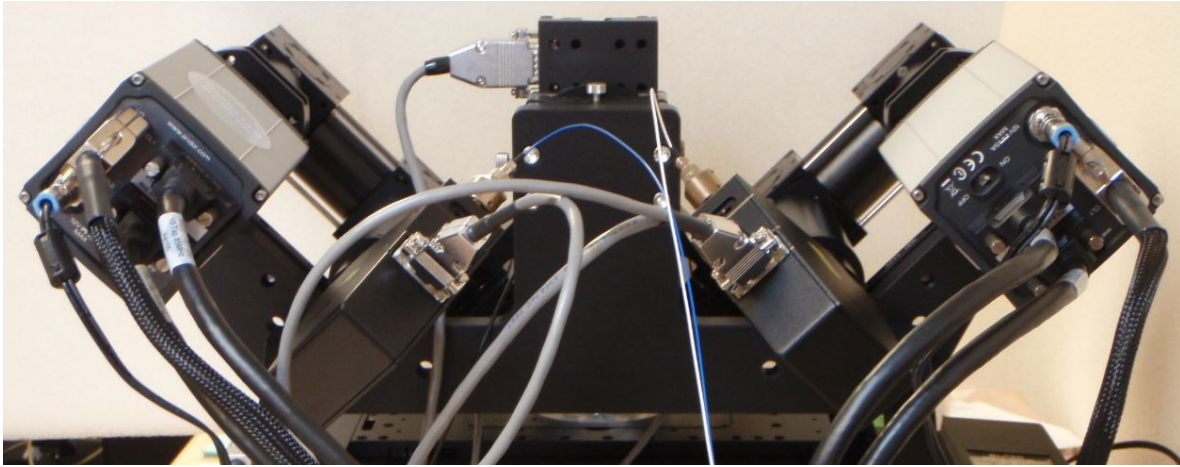


Figure 4: Scanners and Andor Zyla cameras installed in conventional orientation.

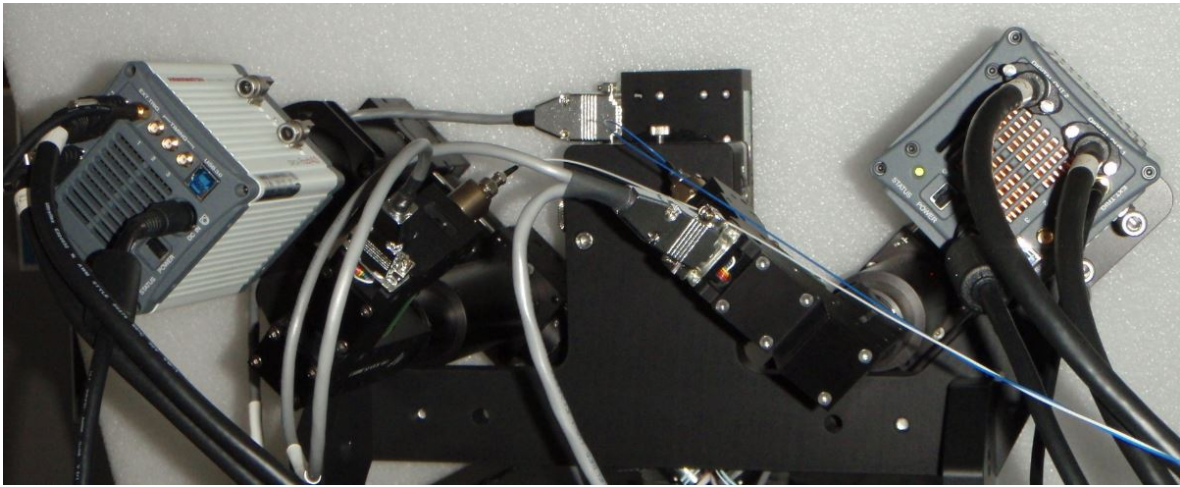


Figure 5: Scanners and Hamamatsu Flash4 cameras installed in conventional orientation.

The scanners should be attached such that the fiber connectors are facing upwards and in toward the center at a 45 degree angle as shown in Figures 3, 4, and 6. Be sure all of the set screws on the lens tubes, cubes, and C-mounts that hold the scanners are snug and the that scanners cannot wiggle around on the mount. After firmly securing the C-mount to the scanner, tilt adjustment is made by rotating the C-mount in the lens tube and securing with the set screws for the C-mount.

2.3 Attach the cameras

The camera tube lenses are fastened to the SPIM-MOUNT with a pair of ring clamps that clamp the tube lenses to the frame. The end of the lens tubes should come to within about 1mm from the mirror cube on the SPIM arm. This allows the entire objective pair assembly to be moved up and down for focus without needing to move the cameras. When the objectives are lowered into position for viewing a sample, the camera tube lenses assembly can be adjusted so that the tube lenses are centered on the mirror cube. Two M8 Allen head bolts hold the lens tube support arms to the frame. By loosening those bolts it is possible to adjust the position of the lens tubes to be centered on the mirror cube.

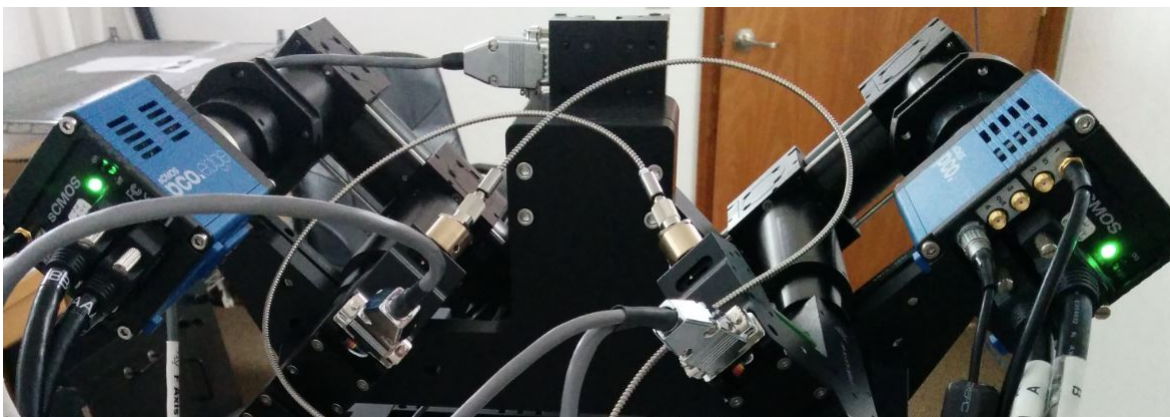


Figure 6: Scanners and PCO Edge cameras installed in conventional orientation.

2.3.1 Camera orientation

The user has a choice of camera orientation. Although other choices are possible, our convention³ is to have the image of the illuminating sheet laser beam appear horizontal with the laser coming from the right objective appearing as coming from the right side of the left-side camera image and vice versa for the opposite objective.⁴ Figure 7 shows the Micro-manger MultiCam image of the light sheet lasers entering a dye solution when the cameras are positioned as recommended. *Actually, this is rotated 180 degrees from the camera orientations shown (but consistent with the view from the bottom camera), a discrepancy that we need to resolve.* For the laser to appear horizontally, the cameras need to be tilted at 45° from vertical. Figures 4, 5, and 6 respectively show Andor Zyla, Hamamatsu Flash4, and PCO Edge cameras oriented in the conventional way.

2.4 Insert the objectives

The method for inserting the objectives was significantly simplified in the piezo mounting scheme introduced in early 2015. Older systems can be upgraded with the new piezo mounting scheme. The main benefit of the new scheme is eliminating the AOA objective adjuster, replacing it by a smooth linear stage, which greatly eases objective alignment. In the new scheme the piezos easily slide out of the arm for easy exchange and cleaning of objectives. The linear adjuster is fixed instead of being moved by the piezo, improving speed slightly. Finally, the bushings include teflon glides to keep them snug.

Refer to the appropriate section to insert your objectives.

2.4.1 With pre-2015 piezos

At one point we recommended screwing in the objectives one at a time, retracting the objective bushings fully to make space, and subsequently returning the bushings to the desired position. However, to lessen mechanical stresses on the piezo actuators, we now recommend assembling the objective with bushing and piezo objective mover (and lateral adjuster if present) separately from the microscope and then attaching it to the SPIM arm mount. This way the objective bushings do not need to be retracted beyond their normal travel, nor do you risk damaging the objective threads as you begin screwing it in slightly off-axis.

Begin by removing one SPIM arm, intact, from the arm mount so you can access the screws connecting the piezo objective mover to the arm mount. It does not matter which side you remove. On the arm that you will remove, detach the electrical connector to the scanner along with its fiber optic cable. Loosen the three set

³which is consistent with the Nature Protocols paper at [doi:10.1038/nprot.2014.172](https://doi.org/10.1038/nprot.2014.172)

⁴Depending on the sample aspect ratio it may be faster to rotate the cameras by 90°. Attaining maximum imaging speed depends on how the camera is rotated relative to the size and shape of the sample being imaged. Camera readout on sCMOS cameras is generally by row, alternating from the center of the image outward.

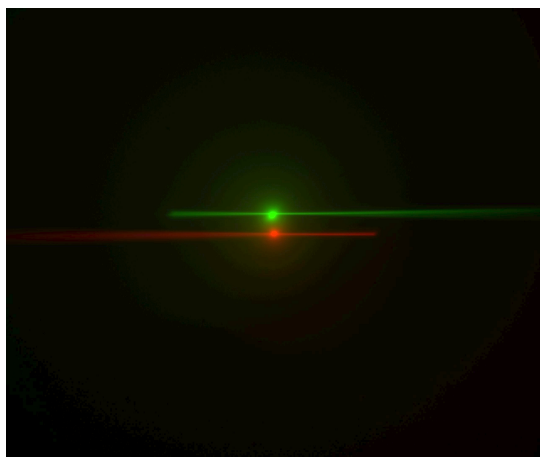


Figure 7: Laser beams seen by cameras in conventional orientation. The green image is from the left-side camera and shows the beam from the right-side scanner entering from the right and eventually striking the coverslip where it stops. The red image is from the right-side camera, shows the beam from the left-side scanner entering from the left. The bright spots are the epi-fluorescent excitation from the scanner on the same side as the respective cameras. The following statement will be understood after going through the alignment: the objective lateral alignment is perfect because the epi and beams exactly overlap, but the camera mirrors need to be adjusted slightly to put both epi spots in the image center.

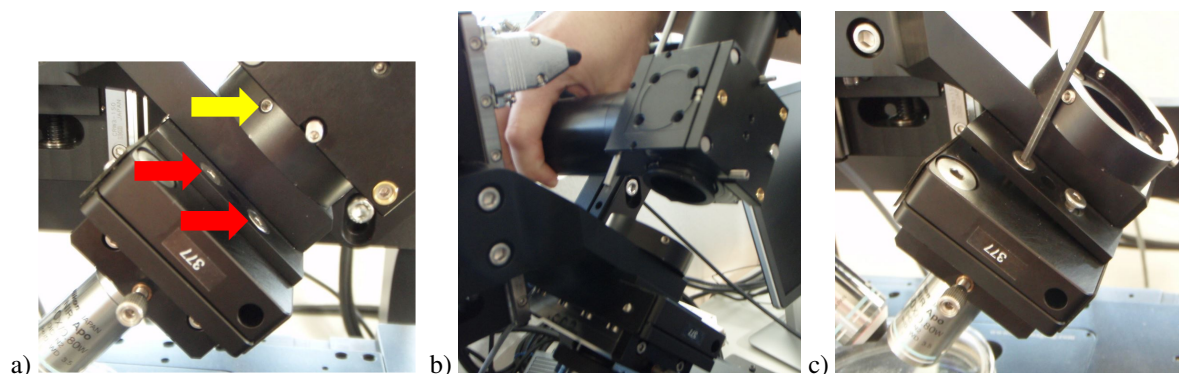


Figure 8: Removing the SPIM arm and piezo objective mover from the SPIM arm mount. a) Close-up view of the screws holding the lower cube and rest of SPIM arm to the arm mount (yellow arrow) and also the screws holding the piezo objective mover to the SPIM arm mount (red arrows), b) Separating the SPIM arm from the mount, c) loosening or removing the screws holding the piezo objective mover to the SPIM arm mount.

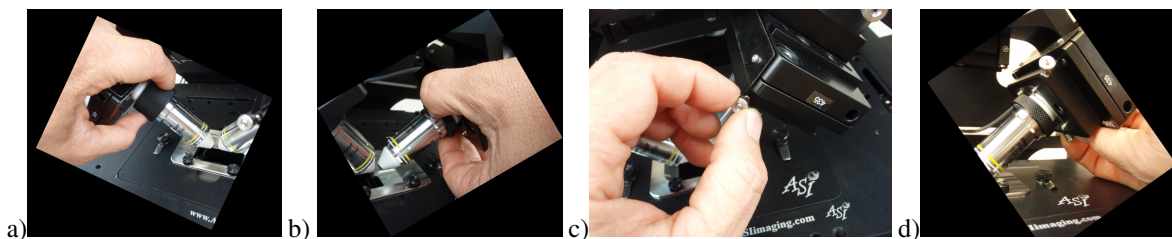


Figure 9: Three orthogonal objective adjusters. a) Left objective focus bushing, b) Right objective focus bushing, c) Lateral objective adjustment screw. The vertical objective position adjuster (d) is used mainly to lower the objective when changing lenses. Its effects are similar to the left objective focus bushing, and hence redundant.

screws around the ring connecting the arm mount to the bottom cube (labels 3 and 9 in Figure 1, or the screw marked with a yellow arrow in Figure 8a). After loosening the set screws you may need to pull slightly on the SPIM arm to disengage the support dowel between the lower cube and the arm mount. Note that the SPIM arm is heavy. Gently set it aside.

Next remove the piezo objective mover (APZOBJ) from the SPIM arm mount as shown in Figure 8c. Loosen the four screws made accessible by removing the SPIM arm; these are the screws marked with red arrows in Figure 8a. It is possible to fully remove the piezo objective mover and everything attached to it from the arm mount, but usually it is sufficient to simply loosen the 4 screws. Next, insert the objective into the bushing on the loosened piezo objective mover. Make sure the bushing is appropriately tightened; the nominal spacing between the objective bushings and the piezo or fixed mount is 5 mm on both sides, which means about 6 mm of bushing thread goes into the APZOBJ or PZMAG-AOA. The objective bushings (RAO-0004 and RAO-0023) thread into their respective piezo device or translatable mount in a smooth but snug fashion. Newer objective bushings have Teflon glides to keep them snug; the earlier version can be retrofitted with judicious use of Teflon (plumber's) tape. ASI no longer recommends using the lock nut provided with early SPIM systems. The gap between the objective bushings and the piezo or fixed mount should be about 5mm on both sides (about 2mm if you have the lock nut installed).

If needed, now is the time to insert the objective on the other side of the diSPIM. It is easy to access the side of the diSPIM that was left intact now that much of the other side is removed (if needed you can raise the upper Z stage).

Re-attach the piezo objective mover with objectives by screwing it into the SPIM arm mounting plate. Finally, reattach the SPIM arm to the arm mount.

With everything reassembled, get the two objectives aligned (co-focused) “by eye” as best as possible using the bushings and the objective adjusters shown in Figure 9. For two Nikon 40x NA0.8 3.5 mm WD objectives, the gap between objectives is 0.2–0.5 mm. Use the adjusters shown in Figure 9 to move the objectives into position until they are visually symmetric from the front of the microscope and aligned forward and backward as well, as shown in Figure 10.

We recommend avoiding use of the vertical objective position screw (Figure 9d) after the objectives have been aligned by eye. Make the vertical adjuster screw is not loose but also not so tight that the lateral adjustment is impossible. Because the lateral and vertical position adjusters are coupled you may need to work the screws against each other.

In further adjustments we will move the objective focus bushings on both sides to focus and manipulate the right objective laterally using the lateral objective screw (Figure 9c).

2.4.2 With 2015 piezos

Inserting the objectives is significantly easier with the 2015 piezo mounting scheme. The piezo actuators and everything attached to them slide in and out of the SPIM arm mount (RAO-0046) using a dovetail mechanism. A new lateral fine adjuster sits between the piezo objective mover and the SPIM arm mount. The objectives are

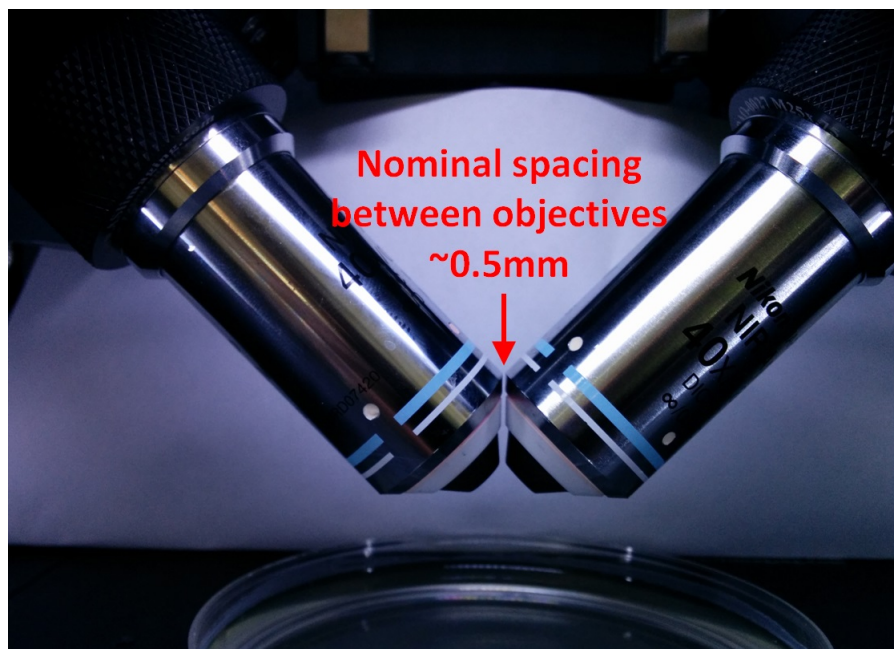


Figure 10: Close-up view of the two objectives when aligned.

brought into co-focus in 3-dimensional space by three orthogonal adjustments: the lateral adjuster and screwing or unscrewing the two objective bushings into the piezo top plate.

To remove the piezos from the SPIM arm mount, first loosen the set screw on the bottom of the arm mount; it is the center screw of the 3 on the side of the SPIM arm mount as shown in Figure 11. This set screw secures the dovetail joint in place. Once the set screw is loosened, the lower (male) side of the dovetail slides out of the arm with a firm pull. Once the dovetail is separated it is easy to insert, remove, or clean the objective (see Figure 12). Both sides of the arm mount have the same mechanism, though it may only be necessary to remove one side.

Upon re-insertion, the dovetail will “click” into position and then the set screw should be tightened.

With the objectives inserted and everything reassembled, get the two objectives aligned (co-focused) “by eye” as best as possible using the bushings on both sides and the lateral adjuster. When aligned, the objectives will be symmetric when viewed from the front of the microscope and aligned forward and backward as well, as shown in Figure 10.

2.5 Care of piezo objective movers

The piezo objective movers are the most failure-prone component of the diSPIM system. It appears that the piezo actuators can be damaged by external stresses, including as screwing the objective bushings in so far that the piezo top plate is moved mechanically (impossible with 2015 piezos or more recent; for older systems be sure to follow the instructions in Section 2.4.1), crashing into the sample chamber, or resting the SPIM assembly on top of the piezo objective movers when assembling the microscope. As a general rule, be careful not to apply external mechanical force on the piezos.

During normal use the piezos are also stressed electronically. Because the electronic stress scales quadratically with applied voltage, being in the extreme negative position (near the sample) wears out the piezo faster than using the piezo near the center position. Of course electronic stresses are part of normal operation, but if the diSPIM is not being used for a long period of time then we recommend either turning off the Tiger controller or else using the **MC <axis>-** command to disable the piezo axes (this reduces the applied voltage to even less than the center position). In Micro-Manager this can be done easily using the “MotorOnOff” prop-

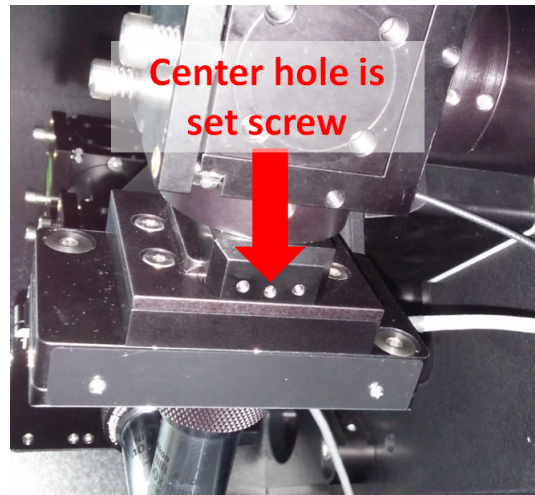


Figure 11: Set screw location for loosening the piezo assembly from the SPIM arm mount.

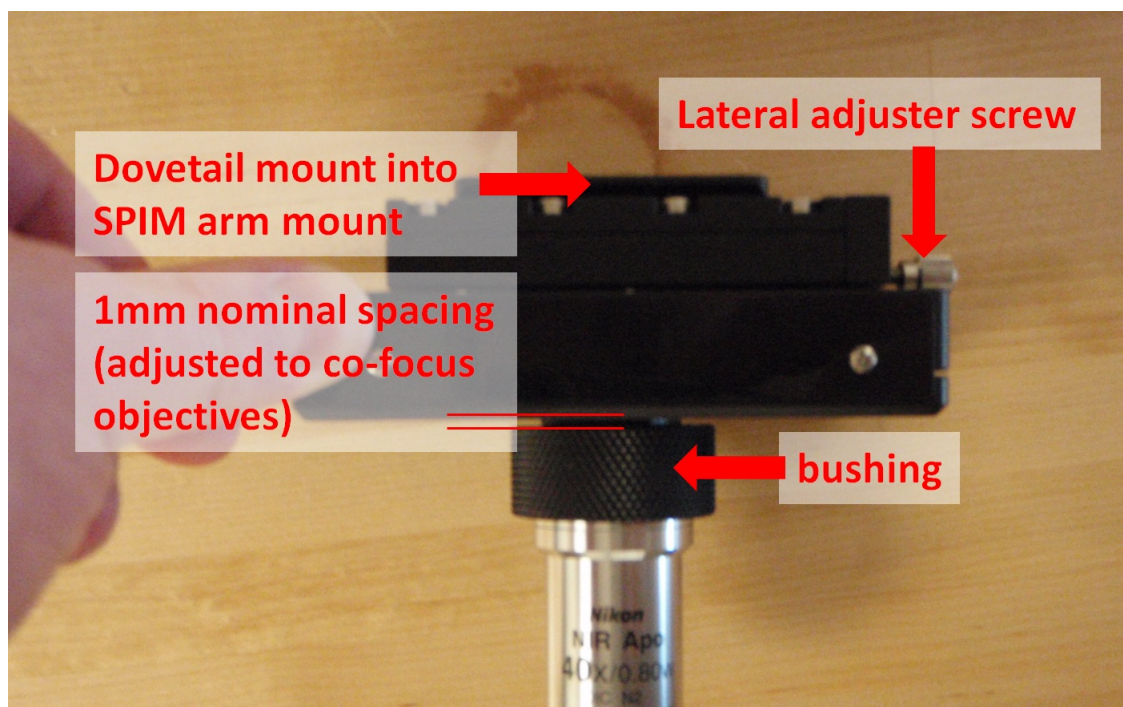


Figure 12: 2015 piezo objective mover with lateral adjuster.



Figure 13: Tiger Controller

erty of the piezo stages, setting its value to be “On” in the System-Startup configuration preset and “Off” in the System-Shutdown preset (see the [Micro-Manager Configuration Guide](#)). Then simply turning off Micro-Manager suffices to put the piezos in a good long-term state. When cared for properly we expect most piezo objective movers to last much longer than the one-year warranty period.

As of December 2014 we recommend 150 μm -travel piezo objective movers with increased flexure width, which reduces the tendency for side-to-side vibration of the piezo top plate in response to other vibrations (e.g. from the camera fans or imperfect isolation from the floor).

2.6 Tiger Controller

The motion components of the diSPIM microscope are controlled by ASI’s modular Tiger controller. The controller should be located near the microscope such that all cables reach and so that the indicator lights on the face of the control units can be seen easily.

A typical diSPIM controller contains the following modules:

TG/COM

Communications card with USB connection to host computer. Supports four-axis joystick/knob pod.

X/Y

Two-axis XY stage card. Also supports a dimmable LED illuminator.

Z/F

Two-axis card for SPIM focus (F) and lower microscope focus control (Z). May also have an LED illuminator.

MICRO-MIRROR

Four-axis micro-mirror controller card. Controls two standard light sheet scanners, or a single scanner with anti-stripping micromirrors. Control logic for synchronizing the light sheet, camera triggers, and piezo motion resides on processor in this unit.

TTL / PLC

TTL buffer card or Programmable Logic card outputs lasers and camera control signals.

PIEZO P

P-axis objective piezo positioner card.

PIEZO Q

Q-axis objective piezo positioner card.

2.7 Connecting cables

Cables to all components are labeled. On the diSPIM there are identical cameras scanners and piezos on both sides so it is important to keep straight which cables go where so that the control software can work as intended.

2.7.1 Tiger Controller

As a safety precaution power off the Tiger controller when connecting or disconnecting cables.

Install all motion control cables to the appropriate Tiger controller module. Be careful that X/Y and Z/F cables are not interchanged. Check serial numbers on the piezo objective movers and controller face-plates to be sure to connect the cables to the correct controller module.

Connect the Tiger USB connection to the host computer.

2.7.2 Programmable Logic Card (or TTL) Card Connections

The Programmable Logic Card, or PLC, was introduced in 2015 and is used for the TTL control of the cameras and lasers. It's outputs should be connected as follows:

1. Path A camera trigger (right-side camera)
2. Path B camera trigger (left-side camera)
3. Not connected (high to indicate ongoing acquisition)
4. Laser side select
5. Laser #1 on/off
6. Laser #2 on/off
7. Laser #3 on/off
8. Laser #4 on/off

Which lasers are #1–4 does not matter, because the mapping between PLC output and a user-defined label is specified in software. In Micro-Manager, define presets using the property “OutputChannel” of the PLogic device (a shutter).

If you have a TTL card (non-upgraded systems shipped in 2014 or earlier), CAM0 and CAM1 are for camera triggers of paths A and B respectively. LSR0 is for the laser on/off control, and LSR1 corresponds to the laser side select. LSR0 and LSR1 may be connected differently if you have a non-standard laser configuration; contact ASI if you need more information.

2.7.3 Light Path A Component Connections

- Left-side scanner is connected to cable end marked **BA**.⁵
- Right-side imaging piezo P is connected to the P-axis Piezo card.
- Right side camera Trigger is connected to PLC #1 (CAM 0 on the TTL card).

⁵Before March 2014 other cables and scanner orientations were used. Contact ASI for new cables.

2.7.4 Light Path B Component Connections

- Right-side scanner is connected to the cable end marked **DC**.
- Left-side imaging piezo Q is connected to the Q-axis Piezo card.
- Left-side camera Trigger is connected to PLC #2 (CAM 1 on the TTL card).

2.7.5 Cameras

Install the camera cards in the host computer according to the manufacturer's instructions, taking care to install any appropriate drivers along the way. In some cases, drivers need to be copied into the Micro-Manager install directory, consult the Micro-Manager wiki or manufacturer's instructions for details.

Connect the camera data cables to the camera cards in the computer. The camera trigger cables should be connected as described.

2.8 Laser and Fiber Installation

There are many possible laser configurations that are theoretically supported. A bare-bones approach uses a single fiber coupled laser which is split with a fiber splitter into two outputs for the two scanners. In this configuration both output fibers have light simultaneously. The scanners act as imperfect shutters ($\sim 0.1\%$ transmission) when steered to their blanking position.

More commonly, users have a laser merge module with dual outputs which controls of multiple laser lines and includes a routing switch that will direct the laser output one of two output fibers. The outputs on the TTL or PLC cards control the lasers. For a single-color setup, connect the TTL laser on/off control to LSR 0 and the fiber-switching signal to LSR 1 on the TTL card. For control of multiple laser lines, the PLC card is required. Generally PLC outputs #5-#8 are connected to the respective laser on/off controls and #4 is connected to the fiber-switching input.

2.9 Install filters, Filter cubes and Mirrors

Dichroic mirrors, emission and excitation filters should be installed in the C60-D-CUBE for each SPIM arm. Similarly, install the right angle mirrors for the camera tubes in the appropriate cubes. Details are shown in Figure 14.

Remove the two thumbscrews the hold the front dovetail mount, C60-DOVE-II, in place. Give a slight tug and twist so the magnets holding the part in place will release the cover assembly from the cube body. Remove the dovetail mount section from the MIM-CUBE-II's covers as shown in Figure 14b. After installing the filters and/or mirrors according to the manufacturer directions, reinstall the internal cube on the dovetail and then reinstall assembled adjustable dovetail mounts, with their appropriate filter cubes or mirrors, on the microscope. Be sure the three kinematic adjusters engage their seats. Press the three magnet buttons to engage the hold-down magnets. Replace the thumbscrews and lightly tighten them.

2.9.1 How to adjust the mirror cubes

The kinematic adjusters are used during alignment to tilt the mirrors in the MIM-CUBE-II's, both for camera mirrors and dichroic mirrors. You will have best results if you follow these steps.

1. Loosen the thumb screws several turns. **Do not manipulate the adjustment screws while the thumb screws are engaged; doing so can strip the adjustment screws.**
2. Grasp the cube body and apply modest pressure to the center of the adjustable face with your thumb to firmly push the kinematic adjusters into their seats. See Figure 23. You may need to tighten the screws slightly so that the adjustment screws "catch".
3. Turn the three adjustment screws as necessary to steer the mirror using a 3/32" Allen driver.

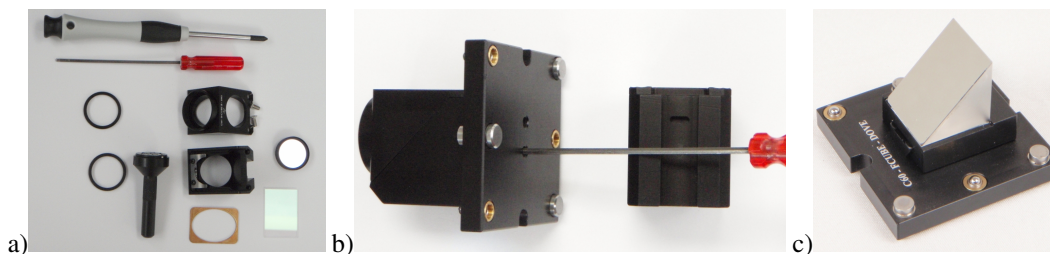


Figure 14: a) Internal filter cube, C60-D-CUBE, shown disassembled and ready for filters. Use the bronze spacer behind the dichroic mirror if using 1 mm thick mirrors. It is very easy to warp mirrors. Barely tighten the mirror retaining clip screws. b) Cube installed on the C60-DOVE-II adjustable cube dovetail mount. There are two set screws on the dovetail mount. One has a small cam end that engages a slot in the internal cube. Align the filter cube slot with this screw and tighten until the cam engages the slot. The cam will allow the cube to be moved slightly up and down. Place in the neutral position and tighten the nylon-tipped set screw to hold the cube in place. c) Right angle mirror installed on dovetail mount.

4. When where desired, lightly snug down the thumb screws and only then release your pressure on the cube face. Slight movement may occur depending on the order and tightness in which you tighten the thumb screws, which can be taken advantage of to make tiny adjustments.

3 The Micro-Manger diSPIM Plugin

Micro-Manager comes with a plugin providing a GUI which facilitates alignment and use of the diSPIM system.

Micro-Manager is a free and open-source software package with many capabilities available at <https://micro-manager.org/>. If you are not familiar with Micro-Manager it is worth reading the [User Guide](#) and [Configuration Guide](#) before proceeding. Micro-manager is continually being improved. If you discover problems there is an easy way to report it to the developers under Help ▷ Report Problem.

The diSPIM plugin is already fully functional but also continually being improved; accessing new features is as simple as installing the latest Micro-Manager version (nightly builds are usually functional and include the latest improvements to the plugin and the rest of Micro-Manager; a major release happens every quarter). If you encounter problems or have feature requests please contact the plugin authors.

3.1 Plugin Overview

In the main Micro-Manager window you can open the plugin under Plugins ▷ Device Control ▷ ASI diSPIM. Keep the main Micro-Manager window accessible; the plugin generally does not duplicate functionality already provided there. The plugin has different tabbed panels for accessing different plugin features for performing various tasks.

The GUI remembers most of its settings from run to run. Some settings are stored on the Tiger controller (e.g. sheet sizes). Before mid-November 2014 some settings were only stored when the plugin was closed before Micro-Manager.

3.2 Before Running the Plugin

Before running the diSPIM plugin, you should create or load a Micro-Manager hardware configuration with all the relevant devices. In the Hardware Configuration Wizard (Tools ▷ Hardware Configuration Wizard) add the Tiger controller (ASITiger ▷ TigerCommHub) and then load all of its peripherals (with the possible exception of the LED shutter). Add the cameras, including creating two devices for the two SPIM cameras for diSPIM systems.

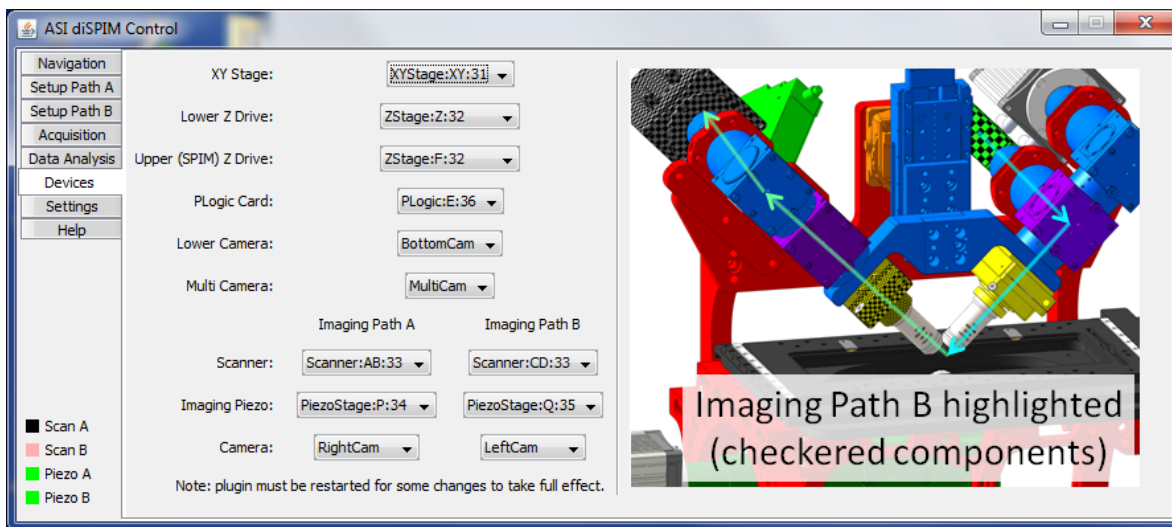


Figure 15: Device tab example.

For diSPIM, you should also create a Multi Camera instance (in the Hardware Configuration Wizard, use Utilities > Multi Camera). You should assign the properties “MultiCam-Physical Camera 1” and “MultiCam-Physical Camera 2” to the two cameras, ideally using the special “System” configuration group with preset name “Startup” as described in the [Micro-manager wiki page](#).

3.3 Devices Tab

Here you assign Micro-Manager devices to roles within the plugin. This is required before using most of the plugin’s features, but is you will only need to re-visit this tab if you change hardware configurations in Micro-Manager or if you launch the plugin without the devices loaded, thus causing the plugin’s settings to be overwritten.

For diSPIM mounted on non-ASI inverted microscope frames the “Lower Z Drive” device may partially work, depending on support in Micro-Manager support for that linear stage.

“Path A” and “Path B” refer to the two light paths as noted in Section 2.7 and shown in the figure of the plugin’s Device tab for reference. The scanner (micro-mirror) and illumination piezo will be one side of the physical microscope, and the imaging piezo and camera will be on the opposite side. For the scanners and piezos care must be taken to match the plugin device assignment with the physical hardware. For ASI’s typical controller configuration, for “Path A” you should select the scanner with axes A and B (usually the left scanner), imaging piezo as axis P (usually right piezo), and the camera which is connected to the right side of the physical microscope. The lower (inverted) Z drive is axis Z, and the upper Z drive (on which the SPIM assembly rides) is axis F. In Figure 15 shows a typical way the Device Tab would be filled out; this is a typical diSPIM configuration except no bottom camera is used.

For single-sided (iSPIM) you can leave the “Path B” devices blank.

Support for the SPIM cameras must be added to the plugin code on a case-by case basis.⁶ Please contact the plugin authors with requests to add support for other cameras. Currently the supported cameras are:

- Andor sCMOS cameras. Use the AndorSDK3 adapter. The bit depth defaults to 12-bit but if you want different the best fix is to add an assignment in Micro-Manager’s “System” group “Startup” preset (see the [Configuration Guide](#)).

⁶The main reason why arbitrary cameras cannot be used is that the SPIM cameras are TTL-triggered during SPIM acquisition but triggered by Micro-Manager during alignment. Thus, the plugin has to know how to switch the cameras between internal trigger mode and external trigger mode, and thus the relevant properties need to be hard-coded into the plugin. Hopefully in the future there will be a camera API call in Micro-Manager to change triggering modes.

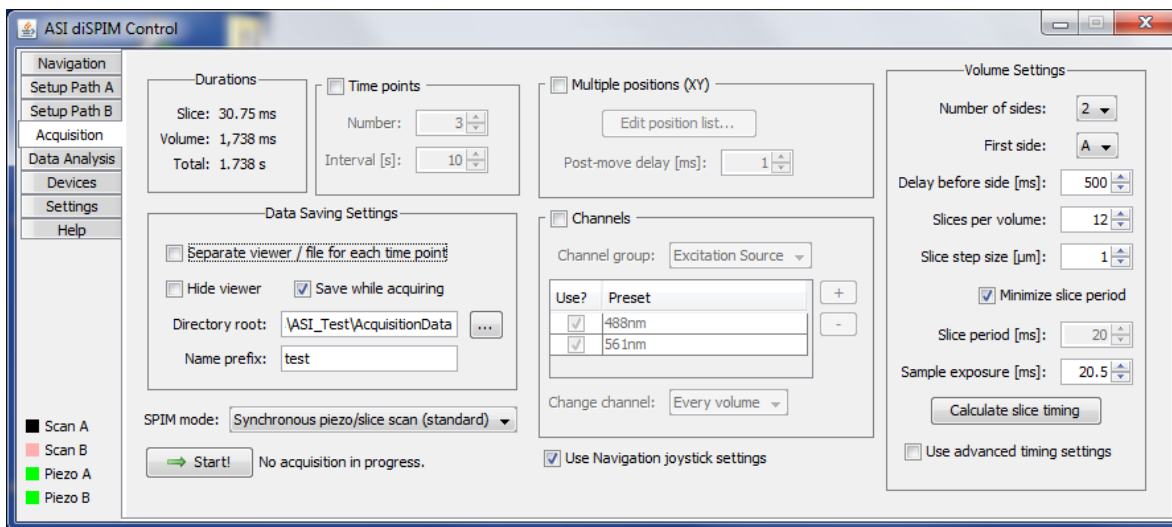


Figure 16: The acquisition tab of the diSPIM plugin with advanced timing settings disabled.

- Hamamatsu Flash 4. Use the HamamatsuHam device adapter. Trigger polarity defaults to negative; the best fix is to add an assignment in Micro-Manager's "System" group "Startup" preset (see the [Configuration Guide](#)).
- PCO Edge. Use the PCO_Camera adapter. Property PixelRate defaults to slow, but for fast frame rates you probably want the "fast scan"; the best fix is to add an assignment in Micro-Manager's "System" group "Startup" preset (see the [Configuration Guide](#)).

3.4 Acquisition Tab

In the Acquisition tab, shown in Figure 16, you set the parameters for the SPIM acquisition and initiate acquisition. This panel is analogous to the Multi-D acquisition window in Micro-Manager, except that the positions of the imaged volume are not specified here. On this tab the joystick and wheels are not functional by default, although a check box is present to enable the same joystick settings as on the Navigation panel.

3.4.1 Setting the 3D ROI

Use the Setup tabs to specify the center slice position. The number of slices per volume and the slice step size determine how far on each side of the center position is imaged.

Use Micro-Manager's usual mechanism to specify the ROI of the two SPIM cameras. Documentation in the [Micro-manager User's Guide](#).

3.4.2 Time Points

Here you specify the number of time points and the interval between them.⁷ The amount of time for the entire acquisition (all time points) is displayed. If the checkbox is left unchecked then only a single time point will be collected.

3.4.3 Data Saving Settings

Here specify where and how the data is saved.

⁷The plugin actually triggers separate single-volume acquisitions in the controller. It is technically possible but not currently implemented to use the controller to set up repeated acquisitions.

- Separate viewer / file for each time point: performs a separate Micro-Manager acquisition for each time point, implying a separate viewer window and separate file. In general it is better to let Micro-Manager save all time points to a single file. Currently a bug prevents proper operation with separate viewers if “Hide viewer” is also selected.
- Hide viewer: suppresses the Micro-Manager viewer. Note that hiding the viewer is cosmetic only, it does not improve the computer’s ability to stream acquisition data to disk because the data collection runs in its own thread.
- Save while acquiring: saves the data to disk; if unchecked then data will be saved in RAM and the user will have the option to save it later. Obviously, for large acquisitions the data must be saved to disk directly.

3.4.4 SPIM Mode

Currently several modes are supported:

- Synchronous piezo/slice scan: standard use, the piezo and sheet move together through the sample.
- Slice scan only: suppresses movement of piezo but still moves the light sheet. It is useful for characterizing light sheet thickness.
- No piezo or slice scan: neither the piezo nor light sheet moves. It is useful for characterizing vibration in the system.

Other modes will be added in the future, for example using the XY stage to scan the sample through a stationary light sheet.

3.4.5 Acquire Button

Obvious function. Click it during an acquisition to abort. The status string informs you which time point is being acquired.

3.4.6 Multiple Positions (XY)

If the box is checked, the plugin will use Micro-Manager’s position list to acquire volumes and multiple locations. For each time point, each position is visited once. See the section “[Position List Dialog](#)” of Micro-Manager’s User’s guide for more details about using the position list.

3.4.7 Channels

The channels feature is patterned after that of Micro-Manager’s Multi-D Acquisition (documentation in the [User’s Guide](#)). It is most commonly used to take volumes with different colors. Using the channels feature requires creating a group of presets which contain all the settings for that channel. Selecting that group in the pull-down menu and then the desired presets.

Simple per-volume channel switching is implemented and is the only choice unless a Programmable Logic Card (PLC) is present in the controller; this mode is generic and can be used with any Micro-Manager group. PLC-based channel switching is hardware-based, requiring extra setup time but then no delay during acquisition. Using the PLC, channel switching is possible on both a per-volume and per-slice basis. In this case the channel group must contain the property “Output Channel” which is used to select the correct laser to trigger; other properties in the channel group, if any, are ignored.

3.4.8 Volume Settings

These settings determine Use the parameters in the “Volume Settings” group to set up the stacks collected as follows.

- Number of sides: 2 for diSPIM, 1 for single-sided.
- First side: which of Path A or Path B goes first.
- Delay before side: wait period after initiating the side (moving the illumination objective into position and the imaging piezo to its start position) before beginning stack acquisition.
- Slices per volume: number of frames to acquire on each side.
- Slice step size: increment of imaging piezo (and light sheet will follow) for each slice, i.e.the Z-stack step size.
- Minimize slice period: when checked the plugin will go as fast as possible.
- Slice period: requested period of each slice, if not minimized.
- Sample exposure: requested illumination time. Currently limited to half-integer values in milliseconds with integer steps.
- Calculate slice timing: calculates and populates the controller’s timing parameters based on the current ROI and settings above.
- Use advanced timing settings: when checked the controller timing settings can be edited directly in a pop-up window. Use this if you want to adjust the values that the plugin computes.

The time required for acquiring each slice, a single volume, and the total acquisition is displayed in the “Durations” box in the upper left.⁸

3.4.9 Advanced Timing Settings

The detailed controller timing settings are hidden by default but can be changed if the “Use advanced timing settings” box is checked. See ASI’s TG-1000 SPIM documentation for timing diagrams and further details.

The advanced timing settings have correspondence to controller settings (see TG-1000 SPIM documentation) as follows:

- Delay before scan: time before scan begins (gives piezo stages time to move/settle before sheet acquisition is started). (**NV X**)
- Line scans per slice: how many one-way beam scans per slice. (**NR X**)
- Line scan period: time for one sweep of the beam (**SAF <axis>**)
- Delay before laser: delay before laser trigger is fired. (**NV R**)
- Laser duration: duration of the laser trigger. (**RT R**)
- Delay before camera: delay before camera trigger is fired. (**NV T**)
- Camera duration: duration of camera trigger. (**RT T**). In current version of the plugin, the camera exposure is set to this value as well for edge triggering, or set to 1 ms for overlap/synchronous mode.⁹

⁸The actual time of the stack (and also the slice period) may be slightly longer due to the firmware implementation details.

⁹In the future we would like to utilize level triggering as well.

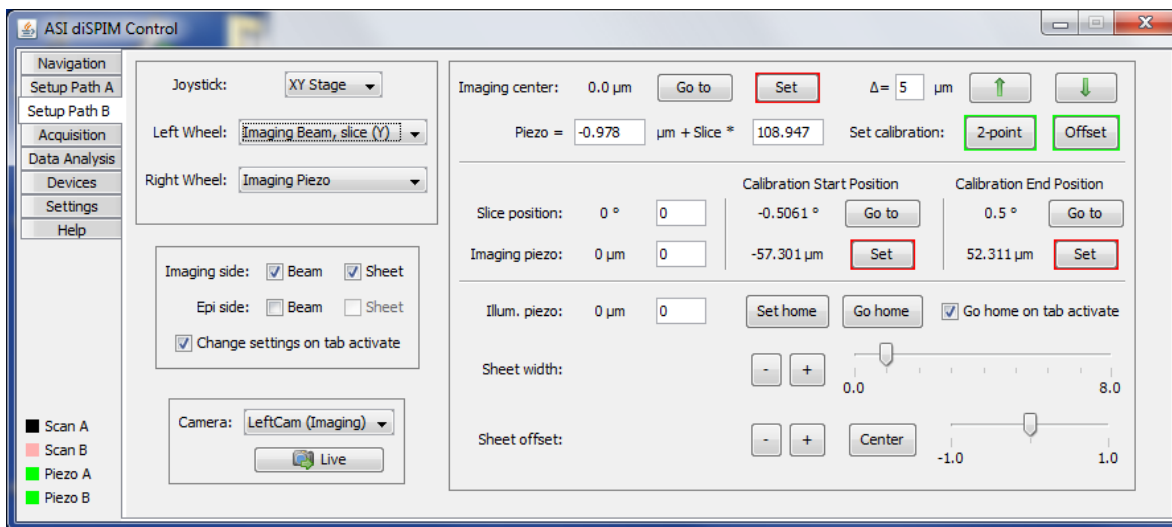


Figure 17: The Setup panel of the diSPIM plugin.

3.4.10 Easy Timing Mode

By default the controller's timing settings are not directly accessible; instead specify the "Slice period [ms]" and "Laser exposure [ms]" in the Volume Settings. Use the button "Calculate slice timing" to automatically calculate the controller's timing parameters. The timing depends on the reset and readout time of the cameras, which is computed according to the ROI as specified using the main Micro-Manager window. Any extra time in the slice is placed before the camera trigger to allow maximum time for piezo settling. The values computed by the easy timing mode are shown in the slice timing settings and can be subsequently modified manually.

The basic algorithm that the easy timing mode uses is described here for the curious. Only one line scan per slice is used. Each period consists of camera readout time, then any extra delay time, then camera reset time, then beam scan time. During beam scan time, the laser is off for the first 0.25 ms and last 0.25 ms of the scan and on otherwise (because the beam scan is limited to increments of 1 ms then the laser exposure time must be a half-integer number of milliseconds). An empirical scanner delay according to its Bessel filter is included (so the scan signal is shifted slightly earlier). Times are rounded to the nearest 0.25 ms except the camera readout and reset times which are always "rounded up" to the next multiple of 0.25 ms. Camera reset and readout times are computed according to manufacturer's timing information and the ROI; they try to account for worst-case jitter. The camera readout time is set to 0 if the "Overlap" on "Synchronous" mode of the cameras is used.

3.5 Setup Tabs

There are two setup tabs, one for each optical path. This tab is essential during diSPIM alignment, and it is also used to set the center position of the imaging piezo (by extension the center of the imaged 3D volume). If you have a single-sided (iSPIM) system then ignore the second tab.

3.5.1 Tab-specific joystick, sheet, and camera controls

Each setup tab has an independent set of joystick/wheel controls, beam controls, and camera control. These settings belong to the tab; e.g. the joystick settings can be separately specified for the two different Setup tabs. The joystick settings become active when switching to the tab. The beams settings on this panel will be automatically set on changing to this tab if the "Change settings on tab activate" box is selected, otherwise the beam settings will remain the same as they were before switching tabs. The beam control uses the scanner "blanking" feature, so it assumes that both scanners have input light (e.g. a passive splitter between the laser and

the scanner). Unless the “No change” option is selected, the camera will automatically be set to the specified camera when the tab is selected.

Similar controls exist on the Navigation tab.

3.5.2 Imaging center and calibration display

The center position for the imaging piezo is shown and can be changed on the top line of the right side. When the “Set” button is clicked, the current imaging piezo position is selected as the new center. This setting is crucial for acquisitions, but can be ignored during alignment.

The up and down arrows in the far upper right of the Setup tab move the piezo and slice together according to the calibration relationship. The amount of piezo travel per button click is set just to the left of the arrow buttons. This is useful for checking whether the calibration relationship is correct, and also for moving through an actual object of interest to make sure that acquisition settings are correct.

3.5.3 Position display and sheet control

The position of the slice is shown, along with the positions of both the imaging and illumination piezos. You can enter a specific position for the axis to move to using the white-background text entry boxes.

For single-sided operation there is no piezo stage on the illumination side. For diSPIM, the illumination piezo has a special “home” position where it goes while being used for illumination (recall that the imaging piezo will be moving along with the slice position). The illumination piezo will automatically go to this home position when the tab is selected if the “Go home on tab activate” is checked.

The sheet width and offset affect the sheet dimensions, i.e. the width and center position of the illuminated area. The “+” and “-” buttons change the value in small amounts; the sliders are easier to use for making large changes.

3.5.4 Setting piezo vs. slice calibration

After alignment is complete the scanner movement in the slice direction needs to be cross-calibrated with the imaging piezo. During volume acquisition, they will move together through the sample to create a stack of images.

Both the piezo objective movers and micro-mirror-based scanners have decent intrinsic linearity, so it suffices to identify two distinct locations where the piezo is focused on the light sheet and compute a linear relationship between the scanner position and imaging piezo. The plugin identifies these as “Calibration Start Position” and “Calibration End Position”.¹⁰ After these two special positions are set using the red-bordered button, click the green-bordered button labeled “2-point” (originally “Compute piezo vs. slice calibration”) to populate the slope and offset of the calibration relationship. The computed values can be manually tweaked if desired. The slope and offset will be used during acquisition along with the (piezo’s) imaging center position to move the piezo and sheet together through the sample while the image stack is acquired.

During normal operation the offset will drift slightly, however the slope will remain relatively constant. Clicking on the green-bordered button labeled “Offset” will adjust the calibration offset without changing the slope. The new calibration offset is based on the *current* position, so the beam should be in focus when you click it.

The recommended procedure to set the calibration start and end positions is as follows:

1. Introduce a fluorescent dye or bead sample where the sheet can be easily visualized, in particular whether the imaging piezo is in focus.
2. Make sure the upper Z stage and illumination piezo are in good positions.
3. Move the imaging piezo to what will be approximately one end of the image. For example, if you are imaging an object that is 50 μm across (and the imaging center is close to zero), move the imaging piezo to 25 μm . You can do that by manually entering the imaging piezo’s position.

¹⁰It does not matter which of the two points is “start” and which is “end”.

4. Use a joystick knob to move the scanner's slice position until the beam is in focus. In a dye solution this is easiest when the sheet is turned off, or with a bead slide it is easiest when the sheet is turned on.
5. Click the red-bordered "Set" button under "Calibration Start Position".
6. Move the imaging piezo to the other end of the imaging piezo's normal range, e.g. $-25\text{ }\mu\text{m}$ for this example.
7. Use the joystick knob to move the scanner's slice position again until the beam is in focus.
8. Click the red-bordered "Set" button under "Calibration End Position".
9. Click the "Go to" buttons for both start and stop to make sure that the imaging piezo and scanner's slice position are correct (i.e. beam in focus) at both points.
10. Click the green-bordered "Compute piezo vs.slice calibration" button to compute the slope and offset of the calibration relationship.
11. Check the computed calibration using the up and down arrows in the upper right of the Setup tab (you may need to increase the increment before doing this).

3.6 Navigation Tab

The Navigation tab (Figure 18) allows the user to easily view and change the position of all ten diSPIM axes. The position of all available axes are visible. By default positions they are updated once per second; see the Settings tab to enable/disable updates and change the update interval.

Below the tab names there is a "quick glance" indication of positions to avoid needing to return to the Navigation tab just to make sure an axis is centered. The color code is as follows:

- Gray = no device
- Green = centered
- Orange = near center
- Red = far from center
- Pink = making sheet (scanner only)
- Black = beam turned off (scanner only)

Like the Setup tab, there are tab-specific joystick, beam, and camera controls. See Section 3.5.1 for details. You can enter a specific position for the axis to move to using the white-background text entry boxes to the immediate right of the current position.

The white-background text entry boxes between the "-" and "+" buttons specifies an increment that the "-" and "+" buttons will move each click.

A button is provided to move each axis to the zero position. It is very useful to move the upper and lower Z axes to the imaging position, then use the "Set 0" button to set that position as zero so it can be easily returned there with a single button click.

Finally, clicking the "Halt!" button will send a command to the Tiger controller to stop the movement of all axes; this is useful mainly if a crash is about to happen.

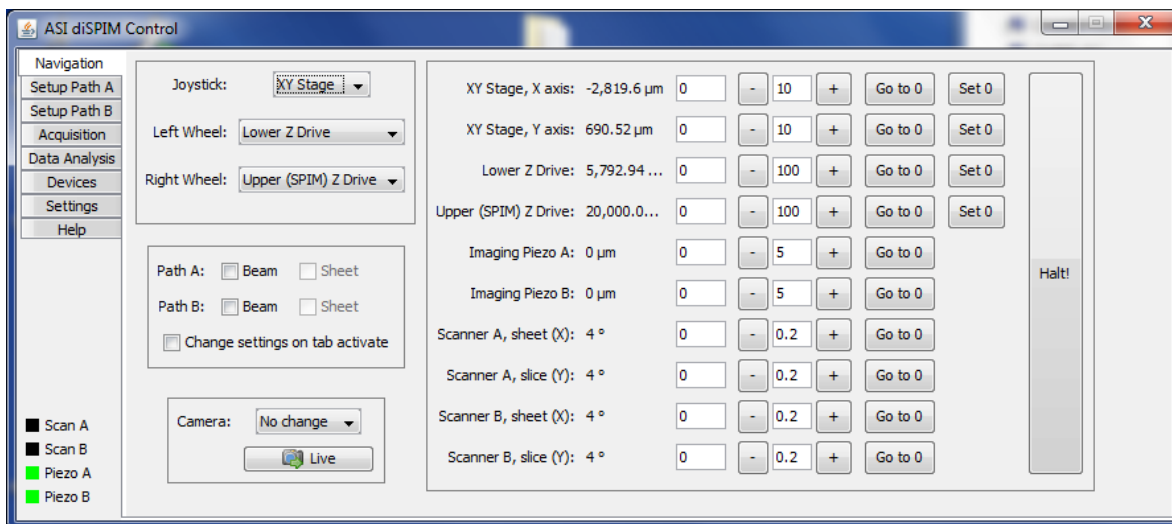


Figure 18: The Navigation panel of the diSPIM plugin.

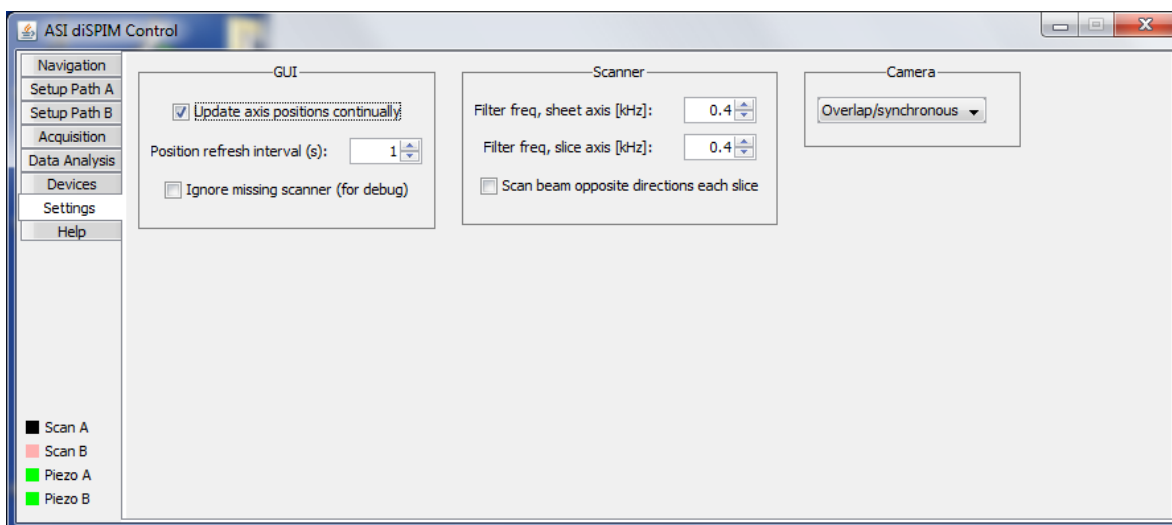


Figure 19: The Settings panel of the diSPIM plugin.

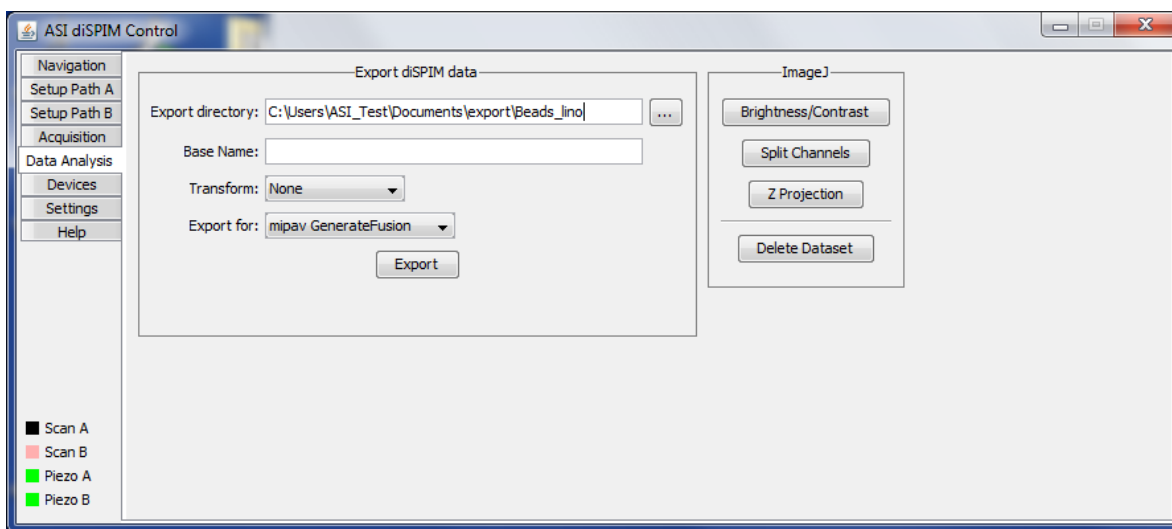


Figure 20: The Data Analysis panel of the diSPIM plugin.

3.7 Settings Tab

The Settings tab (Figure 19) contains various settings not belonging elsewhere.

The micro-mirror drive card has adjustable Bessel output filters to protect the filter from being driven near its mechanical resonance (usually > 2 kHz). Settings as high as 0.8 kHz are usually acceptable, and in general the shorter the scan period (and faster frame rates) the more this matters.

The camera triggering mode is specified here. For Andor Zyla and Hamamatsu Flash4 cameras there is the possibility to have consecutive triggers determine the start and end of a image capture; this is termed respectively “Overlap” and “Synchronous” by the manufacturers. Because the image sensor is simultaneously read out and also reset to begin the subsequent exposure, the camera overhead time is reduced and it allows for faster camera frame rates.¹¹ The same mode is not available with the PCO Edge, however, a new exposure can begin shortly after the readout starts which we refer to as “Pseudo Overlap”.

3.8 Data Analysis Tab

In the future we would like to include the ability to manipulate the acquired data including registration of the two views and joint deconvolution directly in the diSPIM plugin. At present the Data analysis tab (Figure 20) allows exporting the acquired data to the format required by the MIPAV GenerateFusion plugin and Fiji’s Multiview Registration plugin. In the latter case, the XML file that would normally be generated in the “Define Dataset” sub-plugin is performed during data export.

Also in this tab there are some buttons to access commonly-used ImageJ commands. They are simply shortcuts to the ImageJ commands, so they operate on the top-most image window.

4 Set the Stage Limits

When first setting up your microscope you should set the limit magnets on the motorized stages to prevent crashes that can break coverslips or worse. This is especially important when using the $24\text{ mm} \times 50\text{ mm}$ coverslip chamber, where there is not much lateral travel room and the coverslip glass is easily broken. Although the TG-1000 firmware supports software-defined limits, setting hardware limits using the provided magnets is the most

¹¹ $t_{\text{frame}} \approx t_{\text{global}} - t_{\text{readout}}$ with overlap mode, $t_{\text{frame}} \approx t_{\text{global}} - 2t_{\text{readout}}$ otherwise

foolproof way to prevent crashes.¹² The limit magnets move with the stage body; when the magnet passes the Hall effect sensors affixed in the stage body the firmware detects a limit condition and stops the stage.

There are several methods of seeing whether the stage is at a limit. One method is to continually query the limit condition over serial, e.g. using the **RS [axis]-** command; the controller will reply with **U** or **L** for upper or lower limit respectively. Some programs, such as Advanced Serial Port Monitor, allow sending such a command repeatedly for easy monitoring. Another method is to watch the LED of the corresponding drive card on the controller front panel; the LED will flash rapidly when one of its axes is at a limit. Third, a feature is planned for the new version of ASI Tiger Console where an audible beep can be heard when the axis is at a limit.

4.1 Set the upper Z stage limits

The lower limit of the upper Z stage (the LS-50 that raises and lowers the SPIM assembly) is the most important limit to set, and also the most difficult. For the typical Nikon 40x SPIM objectives, the objectives nominally sit approximately 0.3 mm above the cover slip, so the limit must be set within 0.3 mm accuracy to allow the needed range while preventing a crash. To set the lower limit of the upper stage:

1. Prepare a coverslip with a feature on the upper surface (e.g. make a small dot with a permanent marker) and place it in the sample holder.
2. Use the lower objective to focus on the top surface of the coverslip (e.g. focus on the dot). If you cannot focus on the top coverslip surface, the limit magnet is probably set too conservatively and you can move it upwards.
3. Gradually lower the SPIM assembly (e.g. using the joystick or knob set appropriately in the Micro-manger diSPIM plugin Navigation tab) until reaching the coverslip, watching carefully for the moment that the objectives touch the coverslip by noting the change in focus in the bottom camera.
4. While monitoring the limit condition (see Section 4), move the appropriate limit magnet (the top one on the LS-50, very easily accessible) until the limit condition is reached. There is a range of a few mm over which the limit condition is reported; adjust so that the magnet is on top end of that range, i.e. where the controller reports a limit condition but moving it slightly higher takes it out of limit condition.
5. Check that the hardware limit stops the motion appropriately by moving a mm or so up and then back down the same amount (e.g. by issuing the **R F=10000** command followed by **R F=-10000** or dialing back and forth with a control knob). The stage should stop itself slightly short of the prior position, with the objectives nearly touching the bottom of the sample holder (get the position by issuing the command **W F** before and after the move).

4.2 Set the XY stage limits

It is also important to set the limits of the XY stage to prevent the objectives from crashing into the sides of the sample holder, which will perturb objective alignment. To set the limits of the XY stage:

1. Start with the SPIM assembly lowered down to its limit position as set in the prior sequence. When the XY stage is moved too far the objectives will crash into the sides of the sample chamber.
2. Move the XY stage (e.g. with the joystick) until the objectives are just about to crash (or barely crashing) into the side of the sample chamber. Query the appropriate limit status of the appropriate axis (e.g. **RS X-** or **RS Y-**) and move the limit magnet until the limit condition is barely reached. You may need to turn the limit magnets around so the “dots” are facing each other. Repeat for all 4 limits.
3. For all of the four directions, check that the hardware limits stop the motion before the objectives crash into the sample holder using the joystick with the fast speed setting.

¹²An alternative approach to setting the limit magnets is to use the software-defined limits accessed using the **SL** and **SU** commands. Although software-defined limits persist when the controller is powered down, they will be lost if the firmware needs to be updated or reset.

4.3 Set the lower Z stage upper limit

This step prevents the lower Z stage from damaging itself, which can happen in the lower LS-50 motorized stage is lowered so much that the attached objective mount crashes the cube that is attached to the LS-50.

This step does not apply to SPIM systems mounted on non-ASI inverted microscopes. For ASI inverted microscopes this limit magnet is generally set during factory assembly.

To set the upper limit¹³ of the lower Z stage:

1. Gradually lower the lower objective (e.g. using the joystick wheel) until there is a few millimeters of space between the cube mounted to the side of the lower stage and the lower objective holder.
2. While continuously monitoring the limit condition (e.g. **RS Z-**), move the appropriate limit magnet (the top one on the LS-50) until the limit condition is reached. Adjust so that the magnet is on the top end of the range. If needed, the magnet can be flipped around so that the set screw doesn't attach in the region where the magnet track is expanded.
3. Check that the limit stops the motion appropriately by moving a mm or so up and then back down the same amount (e.g. by issuing the **R Z=10000** command followed by **R Z=-10000**). The objective should stop itself slightly short of the prior position, well before a crash could occur.

4.4 Set the lower Z stage lower limit

This step is less critical because the lower Z stage (the LS-50 that raises and lowers the inverted objective) is unlikely to be moved around much, but it is still recommended to set its hardware limit to prevent crashing the objective into the bottom of the sample chamber. The limit can be set anywhere between the focus point of the lower objective in the sample and where it touches the sample chamber bottom, typically 1 – 3 mm depending on the working distance of the lower objective.

This step does not apply to SPIM systems mounted on non-ASI inverted microscopes.

To set the lower limit¹⁴ of the lower Z stage:

1. Raise the SPIM assembly up a few mm from the coverslip in the sample holder so the subsequent steps won't break the coverslip.
2. Gradually raise the lower objective (e.g. using the joystick wheel) until the sample holder begins to move upwards, being pushed by the lower objective. Watch the sample holder from the side to see its vertical movement. Lower the objective until the sample holder is stationary again.
3. While continuously monitoring the limit condition (e.g. **RS Z-**), move the appropriate limit magnet (the bottom one on the LS-50) until the limit condition is reached. Adjust so that the magnet is on the bottom end of the range. If needed, the magnet can be flipped around so that the set screw doesn't attach in the region where the magnet track is expanded.
4. Check that the limit stops the motion appropriately by moving a mm or so up and then back down the same amount (e.g. by issuing the **R Z=10000** command followed by **R Z=-10000**). The objective should stop itself slightly short of the prior position, but still very close to the bottom of the sample holder.

5 diSPIM Alignment

5.1 Overview

Before acquiring data the optical paths of the diSPIM must be aligned. This is the trickiest thing for new diSPIM users to learn. If the system is not disturbed then the alignment can last for months. Changing objectives, crashes, filter changes, and so forth will usually require at least partial realignment.

¹³recall that the positive direction is always away the sample for ASI controllers

¹⁴recall that the negative direction is always towards the sample for ASI controllers

Adjustment	Position?	Angle?	Comments
Tilt of adjustable mirror	Tiny bit	Yes	Set during scanner alignment and not after
Tilt of main micro-mirror	Yes	No	Set during scanner alignment and not after
Tilt of dichroic mirror	Some	Yes	Position effect varies††
Tilt of mirror in camera cube	Yes	No	
Position of upper Z stage	No	No	Angle varies if scanners are not connected to dichroic cubes

Table 1: How alignment adjustments affect the beam position and angle. ††The dichroic tilt affects the angle at which the beam enters the back focal plane as well as the beam position at the back focal plane. It is possible to compensate by adjusting the scan center but we usually adjust the camera mirrors to compensate instead.

Think of the process of aligning the diSPIM as a giant inward spiral. There are lots of different adjustments to be made, and you will adjust the same thing multiple times as you spiral inward to the perfect alignment. First do a coarse mechanical alignment step using your eye and simple mechanical tools. Next, use the cameras imaging a solution of dye and finally a coverslip with beads to zero in on the perfect alignment.

The scanners are aligned at the factory and are likely to be suitable to use without further adjustment. One of the first checks is to verify that they are indeed not terribly mis-aligned before proceeding with other steps. If the scanners fail this initial check, they should be removed and aligned on the bench using the methods described in Section 5.6.

5.2 Ideal final alignment

Below follows a suggested alignment procedure. However, if you achieve good alignment by following some other means that is OK. Here are characteristics of the final alignment you are looking for, which apply to both optical paths of the diSPIM:

1. epi spot centered on epi camera
2. beam waist centered on imaging camera
3. beam/sheet plane coincident with imaging focus plane near the center of the piezo travel
4. beam perpendicular to camera field of view
5. sheet centered in camera field of view
6. sheet centers in center of bottom camera's field of view (i.e. co-alignment of SPIM objectives with bottom objective)

5.3 Beam position and angle: what affects what?

We are concerned with getting both the beam position and angle correct in the sample. Fortunately, the adjustments to do so are mostly “orthogonal” or independent of each other. The following table can be derived by understanding the lens system from the fiber input of the scanner to the sample and then through the imaging path to the corresponding camera on the opposite side of the microscope. Table 1 shows how various “knobs” affect the beam position and angle.

5.4 Coarse Alignment

The following coarse alignment steps are done by eye so that the beams are easily found in the SPIM cameras for the fine alignment.

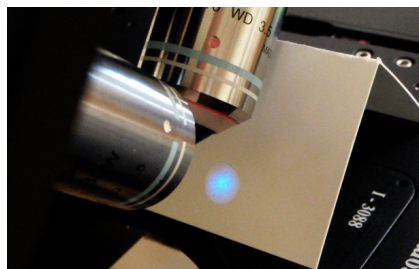


Figure 21: Scanner mirror and iris diaphragm are imaged by the laser on a target in the far-field of the objective. The emerging beam is adjusted to be centered leaving the objective.

5.4.1 Co-align the SPIM objectives

Double-check that the SPIM objectives are approximately aligned as described in Section 2.4.1.

5.4.2 Check rotation of cameras and scanners

Verify that the cameras and scanners are parallel/perpendicular to the SPIM arm cubes as best as possible by eye. Adjust the angles of the devices at their C-mounts as required. With careful sighting along parallel surfaces you can be sure the scanners and cameras are aligned with the rest of the SPIM system to better than 1 degree of rotation. In the long run this will save time having to re-do alignment steps later.

5.4.3 Check course scanner alignment

Warning: be very careful with exposed laser beams; wear appropriate eye protection. Remove the dichroic filter cube assembly (Figure 14b) and place the square alignment target shown in Figure 29b over the cube opening. Be sure that the scan mirror is not being deflected (turn the controller off to be sure), power on the laser, and observe where the laser spot is hitting the target. If the laser spot is well within the first target ring, proceed to the next steps, otherwise proceed to complete scanner alignment in Section 5.6.

Note that it is possible for the scanners to be misaligned in a way that passes this simple test. If in doubt, contact ASI for help.

5.4.4 Adjust dichroic mirror tilt

Re-install the dichroic mirrors so the laser will be reflected into the objective. With upper Z stage (SPIM objectives) raised, tilt the dichroic mirrors in the adjustable cubes (items 9 in Figure 1) as described in Section 2.9.1 so that the laser exits the objectives in a straight line. One trick is to use a 45° surface like a folded business card or part of a C60-D-CUBE with a piece of paper, as shown in Figure 21. Use the cube mirror adjusters to adjust the tilt of the filter cube as described in Section 2.9.1 so that the beam exits the objectives in a straight line.¹⁵ This is a course adjustment only, you will return to adjusting the beam pointing using the cameras later.

5.4.5 Check scanner beam centering

Check the centering of the scanners since it is easy to do at this point. You should be able to “see” the extent of the scanner mirror with the scanner iris fully open. See Figure 21. The center of the Gaussian beam, the center of the iris aperture, and the center of the scan mirror should all be approximately concentric. In practice this is hard to achieve perfectly. If you are not satisfied, proceed to Section 5.6 to improve the situation, or contact ASI for assistance.

¹⁵This process only works when the scanners and lens tubes are hard-attached to the cubes and move up and down with the microscope. If your scanners are fixed to the SPIM frame, then lower the objectives to the sample position before doing this step.

5.5 Fine alignment

For the rest of the alignment we will use the SPIM cameras. The following steps may need to be repeated several times in an alternating fashion until the alignment is complete. It is recommended to first get each alignment relatively close, proceed with the rest of the alignment steps, and then come back to each step to finish; in other words go around the “alignment spiral” a few times not worrying about perfection at first. It is easiest to perform the fine alignment in a dye solution (water with a dip of “Highlighter” pen works well, or a dye like fluorescein). The final steps can also be performed with a 2D sample of fluorescent beads.

You will need Micro-Manager running to have a live view of the camera images. It is helpful to set a relatively long exposure time for the cameras, e.g. 100 ms. Launch Micro-Manager, the diSPIM plugin, and configure the plugin as described in Sections 3.2 and 3.3. You will use the Navigation panel (Section 3.6) extensively, as well as the two Setup panels (Section 3.5).

5.5.1 Co-align the SPIM objectives

Use only three adjusters to manipulate the focus and relative beam positions, the two objective threaded bushings for focus, and the lateral objective position adjuster. See Figure 9. Bring the beams into focus with the imaging objective bushing threads. Alternate between adjusting the two bushings and only move half way to the focus at a time (before alternating sides) to achieve convergence even if there is a bit of play in the objective bushings.

Overlay the images from both cameras using the MultiCam feature and move the lateral adjustment to place the red beam on the red spot, and green beam on green spot. Figure 22 illustrates the procedure. Alternate between the steering adjustment and focus adjustment. If it seems impossible to get a uniform pencil beam, realize that the desired parallel beam might tip it quite far from your current focus point. Go for uniform width first, then focus. As a last resort, see section 5.5.5 to rectify this.

5.5.2 Adjust dichroic mirror tilt

Verify that the the piezos and scanners are in their neutral positions – everything at zero (use the MicroManger diSPIM plugin Navigation tab). Adjust the beam pointing to get the beams pointed exactly perpendicular to the field of view and exactly in the image plane. This was adjusted approximately previously in Section 5.4.4; now adjust it based on the camera images. Use the scanner iris to stop the beam down into a pencil beam. Use the MIM-CUBE-II mirror adjusters (see Section 2.9.1) to make the beam horizontal and in-focus the entire length. Work with one beam at a time. It is not important that the imaging piezo is exactly at 0 at first, you will return to step 5.5.1 again after this step.

Check that the beam plane is coincident with the image plane by adjusting the imaging piezo (e.g. using the joystick knob) to make sure that the pencil beam comes in and out of focus uniformly. It may be helpful to use a LUT (false-color heat map) on a single-camera view to aid in judging uniformity. If the iris is fully opened to create a pronounced beam waist, then the waist position will remain unchanged as you focus and defocus using the imaging piezo. Adjust the mirror adjusters until the beam plane is coincident with the image plane; Figure 23 shows which adjustment screws to use.

After the tilt with respect to the image plane is correct, then adjust the mirror tilt to get the beam horizontal. The grid overlay in the Pattern Overlay plugin in Micro-Manager (Plugins > Acquisition Tools > Pattern Overlay) can be helpful by providing reference lines. Confirm that the tilt adjustment did not tilt the beam relative to the image plane. See Figure 23 for which adjustment screws to use.

5.5.3 Adjust imaging mirrors

Using the mirror adjusters on the two camera mirror cubes, move the epi spot to the center of the camera image. The two sides should overlap in the center. See Figure 22e) & f). You may need to repeat this step again several times as other adjustments slightly disturb this. The crosshair overlay in the Pattern Overlay plugin in Micro-Manager (Plugins > Acquisition Tools > Pattern Overlay) can be helpful by marking the center of the image.

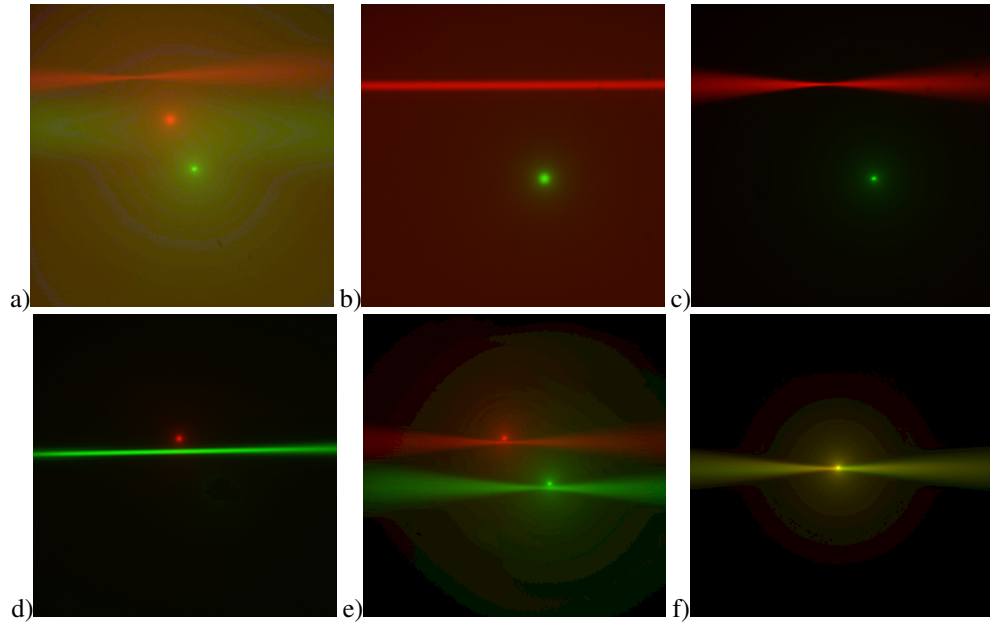


Figure 22: Co-aligning objectives. a) starting out with both beams and epi-spots, nothing very well aligned. b) Look at red beam only with scanner iris closed down all the way so laser is a “pencil beam.” Use adjustment screws on the scanner’s dichroic mirror holder (Section 2.9.1) to make the pencil beam straight horizontally and in focus along the beam. Adjust imaging objective bushing focus as necessary to keep the beam in focus as you make changes to the beam steering adjusters. c) Open the scanner iris and observe the beam waist. d) Switch to the other beam and repeat the alignment of the pencil beam on the other side. e) Now turn both beams on again and use the lateral objective adjuster to bring the red beam to the red spot and green beam to green spot. Notice when everything is in focus, the beam waist is near the epi-spot from the opposite-side beam. You can use this effect to aid in co-focusing the objectives. For example, in a) the red beam is well focused. If you adjust the red-beam *illumination* objective in such a way to move the beam waist slightly to the right, closer to the red epi-spot, the green beam will suddenly become better in focus as well. f) Finally the steering mirrors for the cameras (items 8 in Figure 1) are adjusted to bring the epi spot to the center of the camera frame. First on one side and then to match on the other side.

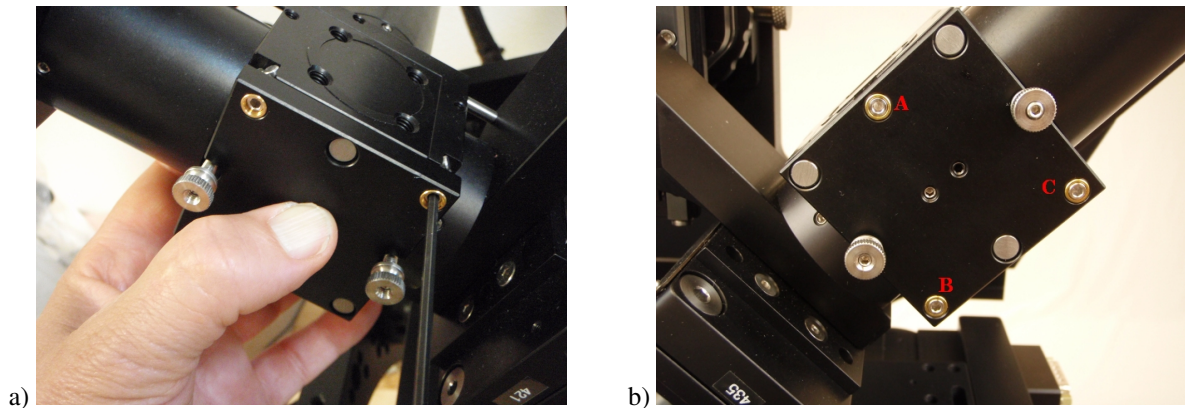


Figure 23: Adjusting the mirror steering screws. Use adjuster A alone, or B and C in tandem, to tilt beam in/out of focus plane. Use adjusters B or C alone, or in opposition, to tip beam horizontally to align parallel with the edge of the camera image.

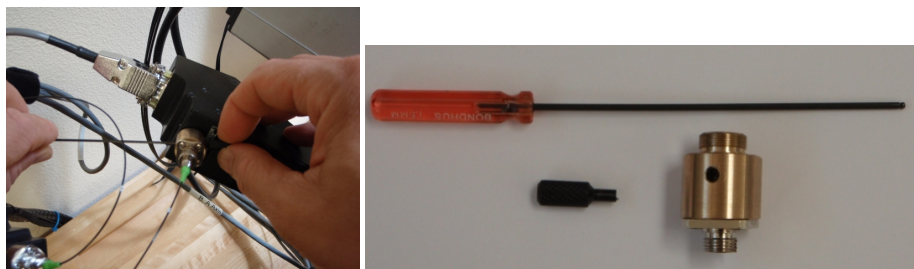


Figure 24: Focusing the fiber collimator to get smallest epi-illumination spot.

5.5.4 Check collimator focus

Open the iris completely to see the focus point; we want that to be in the center of the image. Equivalently, this corresponds to a maximum epi-spot intensity. Insert the fiber collimator focus tool in the opening in the side of the collimator and engage the lip on the lens mount. Turning the tool should change the focus point along the beam (if needed, slightly loosen the retaining set-screw on the opposite side). Only small movements should be needed. See Figure 24. Adjust it so that the beam focus is at the center of the image.

The lens assembly can slip entirely out of the collimator housing if you loosen the set screw without the tool engaged; if that happens you will have to unscrew the fiber collimator from the scanner housing to put it back together.¹⁶

5.5.5 Adjust scanner slice position offset

Usually it is possible to get the objectives co-focused with the piezos near their centered or 0 position. If you have achieved this you can skip the remainder of this step.

If it seems impossible to get the two objectives co-focused with the piezo positions near 0, you have two options: use a non-zero piezo offset or use a non-zero scanner slice position offset. If the sample of interest occupies most of the piezo range, only the later is viable. However, using a piezo offset is a simpler approach. In either case start with the objective bushings moved to a middle position that remains fixed.

Using a piezo offset is straightforward: an offset will be computed automatically by the piezo-scanner cross-calibration explained in Section 5.5.11. Simply you will only need to use start and end points of the calibration curve that are not symmetric about 0.

To use a scanner slice position offset, the piezo-scanner cross-calibration explained in Section 5.5.11 will make the offset appear during imaging, but you should compensate for the offset by moving the imaging mirrors (Section 5.5.3) so that in the middle of the range the epi-spot is centered in the epi camera. Do this by setting the piezo to 0, moving the slice position until the beam is in focus, then adjusting the imaging mirror. Repeat for the other side. This should make it so that the epi spot is overlaid with the beam focus.

5.5.6 Check scanner tilt

Generate a sheet beam using the Micro-Manger plugin “Sheet” check box (Navigation or Setup tabs). Adjust the width of the sheet to fill most of the camera field of view. In a dye solution both edges of the sheet should be well focused near the beam waist. Alternatively, you can move the stationary beam from one side of the sheet to the other using the Navigation panel of the plugin. If the focus is not uniform, then the entire scanner needs to be twisted slightly around its optical axis (loosening set screws and then re-tightening afterward) so that both edges can be equally well-focused. See Figure 25. Check both scanners. If you have to twist one of them, return to the start of the fine alignment.

¹⁶If you do this, make note of the rotational position of the collimator and screw it back the same way. In any case, you might disturb the factory alignment of the scanner so you will probably need to go through the full scanner re-alignment as described in Section 5.6.

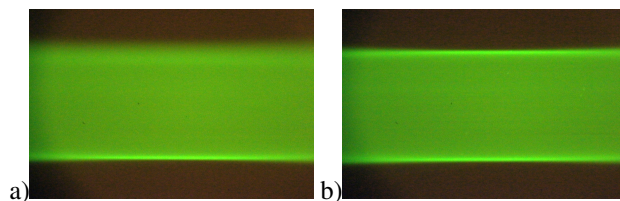


Figure 25: Tilted light sheet (a) corrected (b) by twisting the scanner appropriately on its C-mount.

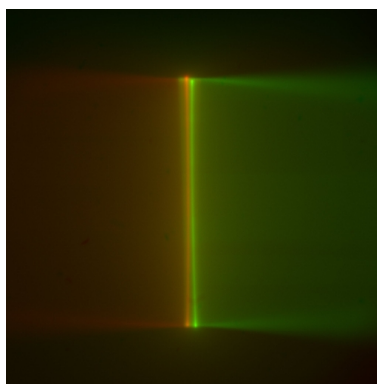


Figure 26: Converging light sheets in dye striking the coverslip. Note the epi-beam line of light corresponding to each sheet. Here the objectives are very close to the bottom of the chamber. The light sheets are barely crossing each other before they disappear when they encounter the coverslip. With the light sheets focuses across the width of the sheet, the epi-beam should appear parallel to the edge of the camera field.

5.5.7 Check camera tilt

Check that the line generated in the epi view while scanning the beam to create a sheet is exactly vertical in the camera image. Because we have verified in the previous step that the scanner is tilted correctly, any remaining tilt must be in the camera. If the epi line is not vertical, adjust the camera until it is and then return to the start of the fine alignment. The easiest way to twist the camera is to loosen the set screw in ABTS-1013 which holds the SPIM cameras' tube lens onto the SPIM assembly. Check both scanners.

5.5.8 Establish coverslip location

Slowly lower the objective pair into the dye solution until the coverslip becomes apparent where the fluorescent beam stops. See Figure 7. The objectives will touch the bottom of the chamber very near the point where the two beams meet in the center of the camera image. Set the Upper SPIM Z-drive coordinate value to zero somewhere near or slightly above this point. In the future, when changing samples you can use the Navigation tab to move the microscope 25 or 30 millimeters and then quickly get back to the coverslip by pressing the "Go To 0" button.

5.5.9 Align bottom objective with SPIM objectives

Locate the image of the laser spots/sheet where it intersects the coverslip in the bottom camera. Bring the laser spots to the center of the camera image by adjusting either the CDZ-1000 XY translator that holds the entire SPIM microscope, or use the bottom-side objective adjuster to center the image (RAMM systems). For larger adjustments, you can add shims between the SPIM mount and the CDZ replacement block for front to back adjustments and shift the dovetail on the LS-50 mount for side to side adjustments. Focus the lower objective and set the coordinate for the coverslip as zero in the Micro-Manger Navigation window. When using the

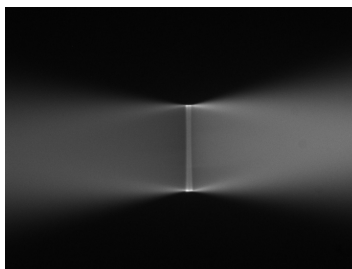


Figure 27: Bottom camera aligned to the centered light sheets shown in Figure 26.

bottom objective for sample spotting, it is useful to know the relative positions of the light sheets in XY and Z compared to the bottom camera image. Setting appropriate Z-references helps in this regard.

5.5.10 Repeat steps 5.5.1 to 5.5.4 at least once

5.5.11 Cross calibrate piezo and scanner movement

This process can be accomplished with either the focused beam in dye, or with a sheet beam using a field of fluorescently labeled objects such as beads on a coverslip or dispersed fluorescent objects in the user's sample. We will describe the process using dye as a natural progression from the previous steps. However, when imaging real samples, you may wish to verify this calibration step fairly often (and redo as needed), in which case you can use fluorescent objects in the sample preparation as target objects rather than the focused beam waist described here.

Use the diSPIM control “Setup Path A” tab. Establish a focused beam in the dye solution. Set the manual controls so that the left knob control the “Imaging Piezo” and the right Knob controls the “Sheet Beam, Slice Position.” Follow the instructions in Section 3.5.4 to calibrate the scanner movement in the slice axis with the piezo movement.

5.6 Aligning the scanners separate from the microscope

Most users will never need to do this step, or will need to do it infrequently. It is possible to make things worse instead of better if you do not know what you are doing.

5.6.1 Overview

The scanners (MM_SCAN-1M) are used to steer the input light beam in the sample. Light sheets are created by simply scanning the beam very rapidly.

We want ensure that the scanner beam is centered in both the image plane and also the objective's back focal plane at the micro-mirror's rest position. Being centered at the back focal plane implies the beam is parallel to the optical axis. For these steps we align each scanner separate from the microscope without driving the micro-mirror, observing the position of the beam through the system.

You should never need to remove the PCB with the micro-mirror on it. The micro-mirror itself is very delicate, and can be damaged by handling (ESD events) and also mechanically (e.g. blowing on it will detach the mirror).

The following materials are required, all included in the scanner alignment kit (SCN-ALIGN-K).

1. collimator adjustment tool
2. spacer tube, 160 mm length (no lens)
3. circular witness targets (Figure 29a has a printable one)

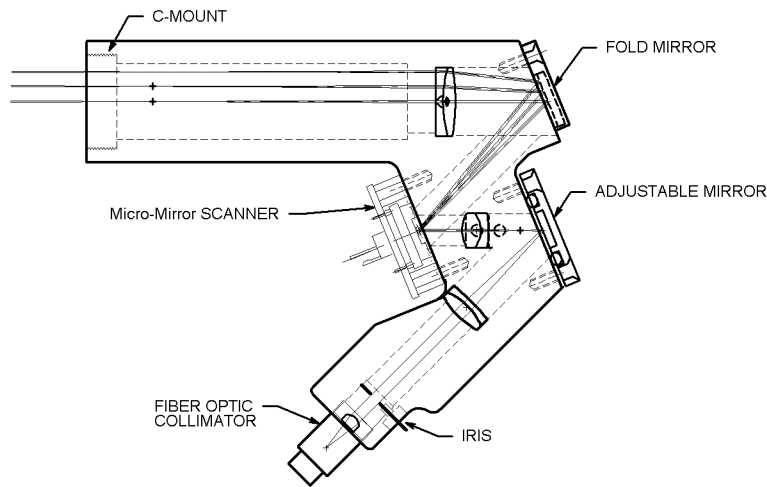


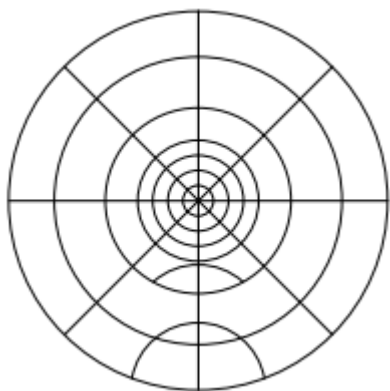
Figure 28: Labeled diagram of internal parts of ASI's Fiber-Coupled Laser Scanner.

5.6.2 Remove the scanner from the microscope

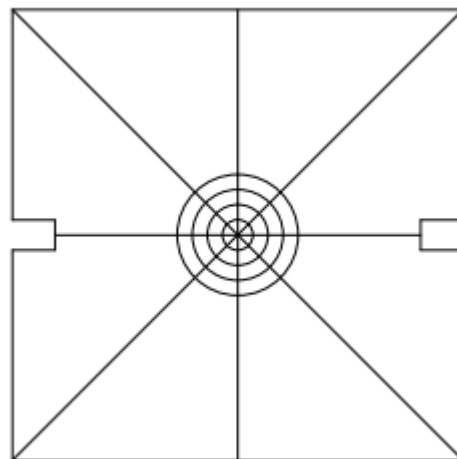
1. Make sure the controller is powered down and then remove the electrical DB9 connector.
2. Optionally disconnect the fiber optic cable.
3. Remove the scanner from the SPIM assembly together with the attached 160 mm tube lens module (C60-TUBE-160 attached via C60-3060-C-MOUNT) and spacer (C60-EXT-TUBE-15).
4. Remove both sides of the scanner cover by loosening the screws on both sides. Set the cover and screws aside.
5. If needed, attach the fiber optic cable.
6. Turn on the light source and verify that a beam exits the tube lens module. **Warning: be very careful with exposed laser beams; wear appropriate eye protection; use low laser power settings.**

5.6.3 Check for beam collimation

1. If needed, connect a tube lens (e.g. the 160 mm tube lens module with C-mount adapter that you removed from the SPIM assembly along with the scanner)
2. Shine a beam through the scanner on a far-away surface (e.g. 10 m away) and make sure the spot size is small, i.e. roughly the same size as it appears on a near surface. You should be able to project a tight 2 mm beam for a long distance.
3. If the beam is diverging, an adjustment needs to be made. This adjustment does not need to be perfect now, as it will be adjusted on the microscope using the SPIM cameras as described in Section 5.5.4. If you need to make this adjustment:
 - (a) Loosen the collimator set screw.
 - (b) Insert the special adjuster tool into the hole and position so that the eccentric tip fits into an internal groove.



(a) Round target. Cut at one of the outer three lines to fit the outside of a tube lens, the inside of a tube lens, and the inside of the scanner C-mount respectively.



(b) Square target. Fits on a cube in place of the cover.

Figure 29: Witness targets useful for alignment.

- (c) While observing the beam on a far surface, turn the adjuster tool (which moves the achromat collimation lens slightly) to make the beam as collimated as possible. The adjustment is not threaded, so each rotation of the tool returns to the same position.

5.6.4 Center the beam at the back focal plane

Other methods are possible, but our recommended approach is as follows:

1. Add a spacer tube to give a total distance of approx. 160 mm from the tube lens module; this is the back focal plane of the objective.¹⁷ Secure the spacer tube so it is concentric with the 160 mm tube lens module. See Figure 30.
2. Tape a witness target on the end of the spacer tube (e.g. Figure 29a, cut appropriately), at the back focal plane of the objective.
3. As shown in Figure 31, center the beam on the witness target by tilting the adjustable mirror of the scanner.¹⁸ The tilt of the PCB with the main scanning micro-mirror should have minimal impact on the beam position in the back focal plane; do not adjust it now. It is easiest to judge the exact beam center if the iris is closed down.
4. If significant modifications are made, re-check step 5.6.3.

5.6.5 Center the micro-mirror on the beam

1. Unscrew the 160 mm tube lens and tube lens adapter so that the scanner is by itself, with the female C-mount exposed. Open the iris fully.

¹⁷We most often use two 75 mm spacer tubes together with an additional 15 mm spacer tube with target attached to the inset rim, giving a total distance of 161 mm. A discrepancy of a few mm is tolerable.

¹⁸If an anti-stripping micro-mirror is installed, tilt the PCB it is mounted on.



Figure 30: Scanner with 160 mm tube lens and spacer tubes.



Figure 31: Adjustable mirror tilted correctly so that beam is centered in the back focal plane of the objective.



(a) Beam not centered on micro-mirror.



(b) Beam centered on micro-mirror.

Figure 32: Views of scanner beam without tube lens, projected on surface about 5 feet away.

2. Shine the beam on a wall, ceiling, or other surface a few meters from the scanner. Look for the circular structure of the micro-mirror, with some pattern around the outside.
3. Push the PCB with the main micro-mirror laterally until the beam is centered on the circular micro-mirror structure. If it is screwed down too firmly, simply loosen the attachment screws a bit, but make sure they are tight again afterward. Figure 32b shows the desired end result.
4. If the lateral range of the PCB is too small (constrained by the screws and holes in the PCB) then the holes can be slightly enlarged using a small file. Usually this will have been done during production at ASI if necessary.

5.6.6 Adjust the collimator angle

Unfortunately we have discovered that the collimator's fiber connector may be slightly off-axis from the collimator threads. We are working on an improved collimator where this adjustment can be done during assembly, but in the meantime the following adjustment can be made (and is being made during assembly as of mid-2014). After centering the micro-mirror on the beam, close the iris. Ideally the central point of light remaining is in the center of the micro-mirror. However, it can happen that the point is not in the center of the micro-mirror. In that case, unscrew the collimator slightly (no more than 360°) and watch the point move. Find the thread position where it is best centered, and use Loctite on the threads to hold the collimator threads in that position.

5.6.7 Center the beam at the image plane

Next, the beam must be centered at the image plane by tilting the PCB with the scanning micro-mirror. One method is to center the beam at base of the C-mount threads, which is very close to the focus point. You can do this using a witness target inserted the C-mount threads, then adjusting the micro-mirror tilt as described in Step 3 below. This is shown in Figure 33. However, it is even easier to use the following approach:

1. Make sure the tilt of the adjustable mirror (or anti-stripping micro-mirror) is correct (Section 5.6.4).
2. Add the 160 mm tube lens and witness target to the end of it. Note that the spacer tube used to adjust the tilt of the adjustable mirror is not present here. The beam position on the witness target at this location is affected by both the adjustable mirror and the PCB tilt, so do not change the adjustable mirror tilt now.
3. Center the beam on the witness target by slightly tilting the PCB with the main scanning micro-mirror. This is done using the 4 screws that hold the PCB to the scanner body, working against a thin piece of elastomer between the micro-mirror package and the scanner. The micro-mirror package should be snug against the elastomer, but make sure that the PCB is not being bent by tightening of the screws. Some interplay with the beam position on the micro-mirror is possible.

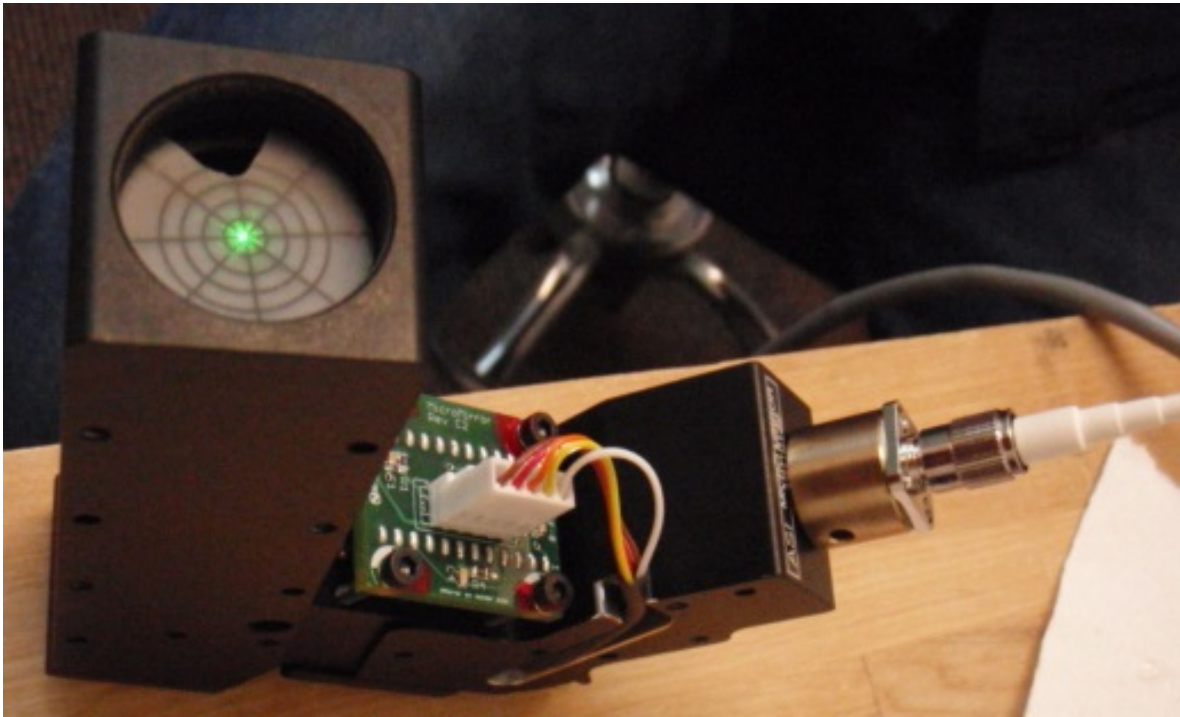


Figure 33: Micro-mirror PCB tilted correctly to center the beam in the image plane at the scanner C-mount.

5.6.8 Repeat until centered

Repeat steps 5.6.4, 5.6.7, and 5.6.5 at least once to make sure that the interactions between adjustments didn't throw things off. When the micro-mirror is properly adjusted, it may be fixed in place with five minute epoxy. This is in general a good idea to prevent the micro-mirror from slipping out of adjustment. During factory assembly epoxy is applied to the micro-mirror and fingernail polish on the screws.

5.6.9 Replace the scanner on the microscope

1. Replace both sides of the scanner cover.
2. If needed, replace the 160 mm tube lens module and C-mount adapter. The scanner should have the tube lens attached.
3. If needed, remove the 150 mm spacer tube. Leave the 15 mm spacer on the end of the tube lens, it acts as a light shield and positions the lens of the 160 mm tube lens approximately one focal length from the back aperture of the objective, for Nikon 40x objectives).
4. Put the scanner and tube lens module back on the microscope and tighten the support ring again so it is firmly in place on the SPIM assembly.

6 Locating and Imaging Biological Samples

7 Troubleshooting

If the system is not working, try to isolate the problem. Useful debugging steps are:

- Power cycle all the components, or the ones in question.
- Make sure each individual component can communicate with its own software. For example, if there seems to be a problem with the cameras then make sure that the camera works with its own software. For the Tiger controller, connect using a serial program like Advanced Serial Port Monitor and make sure communication is OK by issuing the command **WHO** or others.